EFFECTS OF COMPOUNDS OF REGULATORY IMPORTANCE ON THE ACTIVITY OF FUMARATE HYDRATASE

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To my parents

ACKNOWLEDGMENT

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ABSTRACT

EFFECTS OF COMPOUNDS OF REGULATORY IMPORTANCE ON THE ACTIVITY OF FUMARATE HYDRATASE by P.E. Penner. A number of anions have been reported to stimulate or inhibit pig heart fumarate hydratase. Some of these anions, including both substrates, change from activators to inhibitors as their concentration is increased. Bakers' yeast fumarate hydratase was similarly found to be stimulated and inhibited by anions with one or two negative charges, i.e. chloride, acetate, phosphate, sulfate, β-glycerophosphate, glucose-6-phosphate and AMP. ADP, GDP, citrate, pyrophosphate and EDTA were found to inhibit yeast fumarate hydratase at all concentrations of anion and substrate while ATP, GTP, CTP and UTP were the most potent inhibitors of fumarate hydratase studied. Two reaction mechanisms which are compatible with these results are discussed. The inhibition constant for ATP at low fumarate concentrations is 2×10^{-5} M $\pm$ 50% for both enzymes in either direction. The ATP inhibition was not antagonized by AMP or ADP but was partly attenuated by high concentrations of inorganic phosphate and other salts. The inhibition by ATP was antagonized by Mg^{++} and Ca^{++} in a manner indicating that the metal-nucleotide complex has little or no effect on the activity of the enzyme. It is proposed that this permits the enzyme to respond to small changes in the ATP concentration, since, at physiological ATP and Mg^{++} concentrations, the free ATP will vary widely with small changes of total ATP. Many enzymes use $MgATP^{=}$ as substrate, whereas several of the enzymes of glycolysis and the citric acid cycle are inhibited by free ATP,

suggesting that Mg^{++} may serve to mark off the usable ATP from excess or regulatory ATP. This could be a factor in providing a sharp cutoff of ATP production.

TABLE OF CONTENTS

Abreviations

tris	:	tris(hydroxymethyl)aminomethane
P_i	:	inorganic phosphate
PP_i	:	pyrophosphate
EDTA	:	ethylenediaminetetra-acetate
AMP, ADP, ATP	:	adenosine mono-, di-, and triphosphate
GMP, GDP, GTP	:	guanosine mono-, di-, and triphosphate
UTP	:	uridine triphosphate
CTP	:	cytidine triphosphate
AMPS	:	adenylosuccinate

A. INTRODUCTION

a) Purpose:

The purpose of this investigation was to study the effects of various compounds on the activity of fumarate hydratase in order to determine whether specific effects might exist that could be of regulatory significance. Other investigators have observed a variety of effects of anions on fumarate hydratase (1, 2, 3) but have not considered possible regulatory roles for these phenomena. It is now known that many regulated enzymes are composed of subunits, and may exhibit a reversible change in kinetics upon the binding of substrate, various metabolites or hormones. In the light of this knowledge, and the recent discovery of a subunit structure for fumarate hydratase (4), the susceptibility of this enzyme to its ionic environment takes on a new interest. It is possible that this enzyme might be a point of regulation within the citric acid cycle. The study of the regulatory effects of metabolites on enzymes in the citric acid cycle has been much less extensive than the corresponding study on the enzymes of glycolysis. Glycolysis is known to be regulated by metabolites, among which the mono-, di-, and triphosphates of adenine are among the most important.

This study was carried out with two different preparations of fumarate hydratase: a crude preparation from yeast and a crystalline preparation from pig heart. Since individual tissues and organisms may have metabolic requirements which differ, it was

considered important to compare the properties of fumarate hydratases from more than one source. Also, the use of enzymes differing in degree of purification is important, because extensive purification could possibly change the properties of the enzyme.

b) General Approach:

The effects of various inorganic compounds on the yeast enzyme were studied first. The purpose of these studies was two-fold: to allow a comparison of the properties of this enzyme to be made with certain properties of the pig heart enzyme reported in the literature, and to establish a basis for the evaluation of the effects of metabolites on the enzyme. The nucleotides, which are important as substrate and end-product of the citric acid cycle (via the electron transport system) were investigated most extensively. ATP, at low concentrations, was found to inhibit yeast fumarate hydratase so potently that, under the conditions of the assay, an ATP concentration near the physiological level would largely abolish the activity of the enzyme. It appeared likely that some component present in the cell must antagonize the ATP inhibition of fumarate hydratase in order to allow rapid functioning of this enzyme when required. Since magnesium ion is present in the cell sap in concentrations equal to or greater than the ATP concentration, and since divalent cations bind ATP strongly, the effect of divalent cations upon the inhibition by multivalent anions was studied. The effect of

divalent cations in reversing the ATP inhibition suggests a possible relationship between the observed movements of divalent cations and certain physiological phenomena, such as the Pasteur effect.

Hydrogen ion is released from the mitochondria concomitant with magnesium or calcium uptake by the mitochondria, suggesting that pH changes might be an important factor in metabolic regulation. In order to limit this project to a manageable scope, however, and also to permit a comparison of the effects of the various compounds studied, all experiments were done at pH 7.

c) Organization of the Thesis:

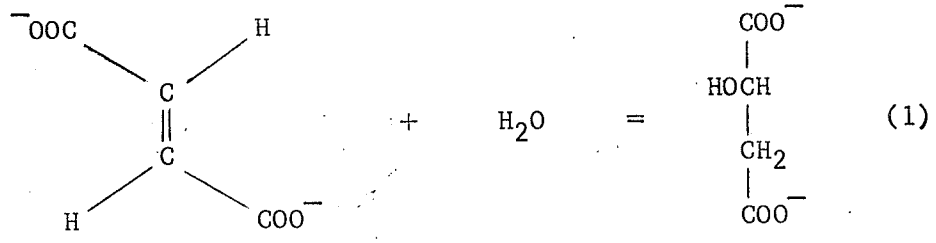
Three main sections, Literature Review, Experimental, and Discussion comprise the body of the thesis. The literature concerning fumarate hydratase deals almost exclusively with the pig heart enzyme. The Literature Review includes, in addition to the properties of fumarate hydratase, certain aspects of current knowledge about regulated enzymes. The Experimental section is divided into two parts, dealing with the experiments performed with the yeast and pig heart enzymes respectively, while in the Discussion both enzymes are treated together in a comparative manner.

B. LITERATURE REVIEW

a) Fumarate Hydratase (L-malate hydro-lyase, EC 4.2.1.2):

i) Discovery: Fumarate hydratase, formerly known as fumarase, was discovered in 1919 by Einbeck, but the existence of the enzyme had been indicated by earlier workers (5).

Fumarate hydratase catalyses the reaction:



(1)

fumarate water L-malate

The reaction is readily reversible: $K_{eq} = \frac{\text{malate}}{\text{fumarate}} = 4.4$.

This reaction is a link in the citric acid cycle of reactions that oxidise fatty acids, carbohydrates, and some of the amino acids. The enzyme is also required for the synthesis of aspartate from fumarate (via malate and oxalacetate) in the urea cycle and in the synthesis of AMP from IMP (6). Fumarate hydratase occurs as a soluble protein and may be demonstrated in homogenates of various tissues: blood, liver, skeletal and heart muscle, molds, bacteria, yeasts, and higher plants (5). Subcellular fractionation studies have shown that fumarate hydratase is at least partly associated with the mitochondria in all tissues examined, and that the extramitochondrial fumarate hydratase is essentially soluble (7) although some activity is associated with the microsomes. This microsomal fumarate hydratase activity was shown to be an absorbtion artifact (7).

ii) Purification and molecular properties: Fumarate hydratase has been obtained in crystalline form by Massey (8) and by Frieden et al (9) who obtained 15 to 20 mg of pure enzyme per kg of pig heart muscle. The most recent fractionation procedure (10) results in a yield of 100 mg of crystalline fumarate hydratase per kg of pig heart.

Crystalline pig heart fumarate hydratase has a UV absorbtion spectrum characteristic of a simple protein; a solution of 1 mg per ml of enzyme has an absorbence of 0.51 at 280 mμ (10). A single symetrical peak is obtained in the ultracentrifuge and in electrophoresis (11). On the basis of sedimentation velocity-diffusion measurements the molecular weight is 220,000 (9) while analysis by sedimentation equilibrium yields a value of 194,000 (4). The difference between these values is probably due to error in the diffusion constant used by the earlier workers (12).

Zone electrophoresis of extracts of torula yeast has indicated the presence of two fumarate hydratases with different electrophoretic mobilities (13). This suggests that two kinetically different enzymes may exist in the same tissue, possibly in two compartments in the cell, although fractionation of subcellular components was not attempted by the authors.

The results of studies by Kanarek et al (4) show that pig heart fumarate hydratase is composed of four subunits of molecular weight 48,500, all of which are either identical or very similar to each other. The molecular weight of the subunits was obtained by sedimentation equilibrium in 6 M guanidine hydrochloride.

iii) Kinetics and mechanism: Much information is available about the fumarate hydratase reaction and a theory aimed at describing the mechanism must take all this knowledge into account. Studies on fumarate hydratase have been done along several lines of investigation which will be dealt with one at a time. The topics to be covered include temperature studies (Arrhenius plots), isotope effects, pH studies, anion effects, substrate activation, and mechanism of the reaction.

1) Temperature studies: Two effects are observed when the rate is determined as a function of temperature. In alkaline solutions the Arrhenius plot has an upward curve as the temperature is increased (14) indicating a higher activation energy at higher temperatures. This discontinuity is distinguished from other discontinuities observed in acid solutions (see below) by the facts that it occurs with either fumarate or malate as substrates, that it is not accompanied by a discontinuity in the K_m, and that the transition temperature varies with the pH from 22° to 32°C. Massey suggests this is due to a dissociation of the enzyme into units of smaller molecular weight as the temperature is increased, these smaller units having a higher activation energy (15).

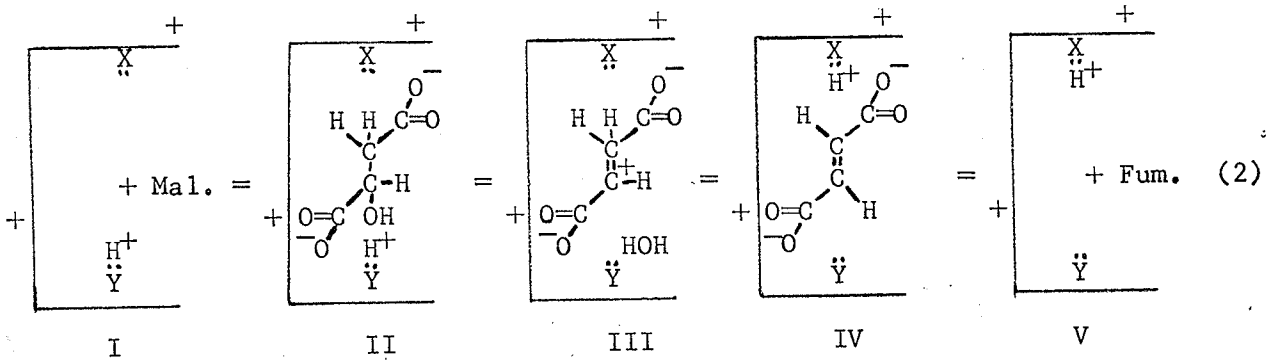
A discontinuity in the Arrhenius plot affecting the forward reaction only is seen in acid solutions. A downward bend occurs with fumarate as substrate (lower activation energy at higher temperatures) at about 18°, this transition temperature being practically independent of pH (14, 15). The change in activation

energy is accompanied by an equal and opposite change in the apparent heat of combination of the enzyme with fumarate, calculated from the effect of temperature on K_m. Inhibitor affinities also show similar discontinuities (2). It seems clear that in acid solution two different enzyme-fumarate complexes exist on the two sides of the critical temperature, both capable of reacting. In neutral solution no discontinuities of either kind are observed.

2) Isotope effects: A review of the studies in deuterium oxide is given by Alberty (11). When fumarate is hydrated in D_2O a single deuterium atom is found on the methylene group of the L-malate, and the removal of this deuterium atom is absolutely stereospecific, i.e. the fumarate formed when this monodeutero-L-malate is dehydrated by fumarate hydratase contains no deuterium. It has been shown that the hydrogen and hydroxyl groups are added to fumarate in a trans manner by pig heart and yeast fumarate hydratase. Two approaches (16), nuclear magnetic resonance spectroscopy, and a stereospecific synthetic approach give evidence that a trans addition to fumarate occurs.

A study of the isotope effect revealed no change in the V_{max} or in the K_m when monodeutero-L-malate was the substrate (17). This is taken as evidence that the breaking of the C-H bond is not rate-limiting, since a 2- to 7-fold decrease in the rate would be expected upon substitution of deuterium for hydrogen if this step were rate-limiting. On the basis of these studies

Alberty concludes that the reaction is not concerted, but occurs in two steps as shown in mechanism 2.



(2)

In mechanism 2 the slow step is between II and III but not necessarily the rupture of the C-O bond as illustrated here. If the slow step preceded breakage of this bond then II and III need not have the order shown in the illustration.

No isotope exchange between the stereospecific hydrogen or hydroxyl O^{18} of L-malate and the solvent could be demonstrated (11). Since there is no exchange, Alberty concludes that the C-H bond must be broken after the rate-determining step in the dehydration of L-malate to fumarate. An intermediate, EX, is required in the mechanism to explain the two observations of the isotope study:

$$E + M \rightleftharpoons EM \underset{\text{slow}}{\rightleftharpoons} EX \rightleftharpoons EF \rightleftharpoons E + F \qquad (3)$$

A small isotope effect was demonstrated by later workers (18). They studied the fumarate hydratase reaction under conditions that were very different from Alberty's experiment (above). The forward reaction (F $\longrightarrow$ M) was studied in D_2O at 17 mM fumarate and a 50% decrease in initial velocity noted. These results, however, do not contradict Alberty's results (above) because

TABLE I

Kinetic Constants for Pig Heart Fumarate Hydratase in Tris Buffer at pH 7

	10 mM tris	20 mM tris	90 mM NaCl +10 mM tris
K_M - - - - - - -	17.3 μM	16.6 μM	113 μM
K_F - - - - - - -	4.7 μM	7.2 μM	80 μM
V_M - - - - - - -	1.19×10^3	1.09×10^3	0.36×10^3
V_F - - - - - - -	$1.5. \times 10^3$	2.1×10^3	1.13×10^3

K_M and K_F are the Michaelis constants for the reverse and forward reactions respectively. V_M and V_F are the maximum initial velocities for the reverse and forward reactions respectively in units of 'molecules per second per molecule of enzyme'.

Alberty did not study the reaction under these conditions.

Evidence for the participation of two groups, acidic and basic, was obtained from pH studies (below). The stereospecificity of the reaction, however, already suggests that the proton must be provided by a specific proton donor on the enzyme molecule shown as X in mechanism 2.

3) pH studies: The variation of enzyme activity with pH has been used by Alberty to determine the pK's of the dissociable groups on the enzyme. The bell-shaped pH-activity curves in the forward and reverse directions show pH optima of 6.2 and 7.6 respectively in 10 mM tris acetate buffer (19); however, these values depend on the composition and concentration of the buffer anion. The simplest mechanism which fits the pH data for the pig heart enzyme at low substrate concentrations between the pH's of 5.5 and 8.5 involves an acidic and a basic group on the enzyme which must be protonated and unprotonated respectively for reaction to occur. Equations for the pH dependence of the kinetic constants have been derived according to this mechanism by Alberty (20). From these equations the kinetic constants for the pig heart enzyme in tris buffer at pH 7 are calculated (Table I).

4) Anion effects: Anions may stimulate the enzyme, inhibit the enzyme, or both. The effects are seen as changes in the kinetic constants (K_m and V_{max}) or as changes in the pH dependence of the initial velocity, i.e. the pH-activity curves. When the bell-shaped pH-activity curve is shifted toward more

TABLE II

Effects of Anions (Literature Review)

Anion	direction measured	approximate effective range*	sign of effect	arm of pH-activity curve affected	ref.**
chloride	M	100 mM	-	acidic	2
bromide	M	100 mM	-	acidic	2
iodide	M	100 mM	-	acidic	2
thiocyanate	M	100 mM	-	acidic	2
"	M	K_i = 14 mM	-		21
"	F	K_i = 50 mM	-		21
acetylene dicarboxylate	(F,M)		-		22
succinate	M	K_{EHI} = 0.32 mM	-	both	3
"	F,M	K_i = 52 mM	-		22
D-tartrate	M	K_{EHI} = 0.34 mM	-	both	3
"	F,M	K_i = 25 mM	-		22
L-tartrate	M	K_{EHI} = 1.3 mM	-	both	3
meso-tartrate	M	K_{EHI} = 0.0029 mM	-	both	3
arsenate	M	50 mM	+	alkaline	2
borate	M	20 mM	+	alkaline	2
citrate	M	10 mM	+	alkaline	2
"	F,M	K_i = 3.5 mM	-		22
arsenite	M	10 mM	+	alkaline	2
selenate	M	30 mM	+	alkaline	2
sulfate	M	25 mM	+	alkaline	2
D-malate	F,M	K_i = 6.3 mM	-		22
trans-aconitate	F,M	K_i = 6.3 mM	-		22
mesaconate	F,M	K_i = 25 mM	-		22
maleate	F,M	K_i = 11 mM	-		22
adipate	F,M	K_i = 100 mM	-		22
glutarate	F,M	K_i = 46 mM	-		22
malonate	F,M	K_i = 40 mM	-		22
glycine	(F,M)	(K_i = 100 mM)	-		22
L-α-hydroxy-β-sulfopropionate	F,M	K_i = 16.5 mM	-		22
phosphate	F	60 mM	+	alkaline	2
"	F	5-130 mM	+		1
"	M	0.33-60 mM	+	alkaline	2
"	M	83.5-333 mM	-	alkaline	2
"	M	5-133 mM	-		1
No effect was produced by acetate, butyrate, crotonate, L-, and DL-aspartate, acetoacetate, mono-, and dimethyl esters of fumarate, the SH-inhibitors: iodoacetate, iodoacetamide, iodosobenzoate, p-chloromercuribenzoate, and chloroacetophenone.					22

*Where the K_i's are not given, the value represents the concentration which produced significant inhibition in the cited work.

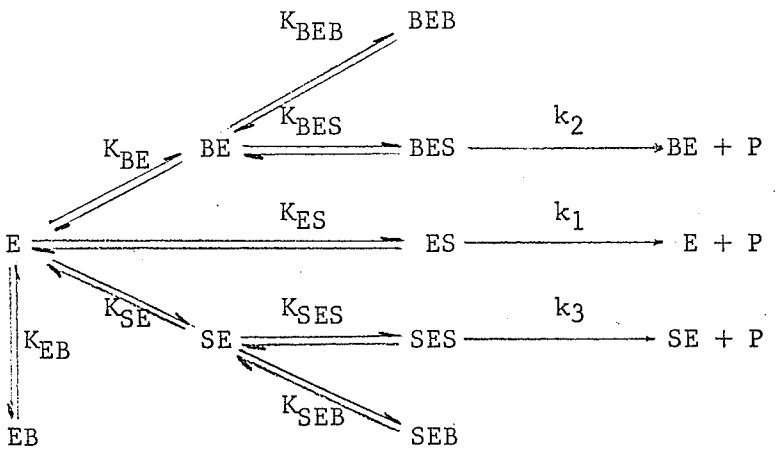
**The conditions of the assay are: 1 - low substrate concentrations, phosphate buffer as indicated; 22 - 60 mM phosphate buffer; 21-high substrate (as in 19) in 33 mM phosphate buffer; 2 - 16.7 mM fumarate or 83.5 mM L-malate (no added buffer); 3 - 10 mM tris acetate buffer.

alkaline or more acidic pH's, stimulation may occur in one pH range and inhibition in another range, or alternately the potency of an inhibitor or a stimulator may vary markedly with the pH of the assay. Massey (2) explains anion effects in terms of the theory (see above) that the dissociation of two particular groups, acidic and basic, determines the activity of the enzyme. Massey suggests that the anions produce specific activating or inhibiting effects by altering the pK's of the catalytic groups of the enzymic site. It is suggested that the anion binding sites are near the active centre of the fumarate hydratase molecule but the theory does not specify in what manner the anions combine with the enzyme molecule. Massey points out that it is not possible to argue, from the experimental results, that only one acidic and one basic group are involved in enzyme function.

The effect of phosphate on the pH-activity curve is entirely confined to shifting the alkaline branch of the curve toward more basic values. This suggests that the basic dissociation constant(s) of the enzyme is affected by phosphate rather than the acidic (2). These results were obtained at high substrate concentrations at a single phosphate concentration and only stimulation was produced by phosphate in both directions of the reaction. Effects similar to those of phosphate were seen with all the activating anions investigated (Table II). The anions that were found to activate thus appear to alter the basic dissociation constant(s) while having little effect on the acidic dissociation constant(s). Monovalent ions such as chloride seem to have no effect on the basic dissociation constant, but do affect the acidic dissociation constant, the shift again being toward more alkaline

values. The monovalent ions inhibit fumarate hydratase as seen in Table II. Thiocyanate shifts the pH optimum to the alkaline side, and for this reason it inhibits below pH 7 but activates the forward reaction above pH7(21). Similarly, the effects of anions depend on their concentration. A dual effect is produced by some anions, activation occuring at low concentrations and inhibition at high concentrations of anion, e.g. thiocyanate (21) and phosphate (1). A similar dual effect also occurs with citrate, although in this case the buffer concentration is important in determining whether inhibition or activation occurs. Thus citrate is a competitive inhibitor in 60 mM phosphate buffer (22), but activates the salt-free enzyme (reverse reaction) (2). Unfortunately in some of these experiments only one citrate concentration was used, thus making it difficult to compare the results reported in the literature.

Alberty has derived equations which fit the initial rate data obtained for pig heart fumarate hydratase in phosphate buffers. The equations are derived for Mechanism 4 which postulates two sites on the enzyme molecule, the enzymic site and an activating site.



(4)

Where B (buffer anion) or S (substrate molecule) written to the left of E (enzyme) indicates binding at the activating site, while B or S written to the right of E indicates binding at the enzymic site. Phosphate may bind at both sites; at the activating site it increases the V_{max} and the K_m, while at the enzymic site it behaves as a competitive inhibitor. It is possible that Mechanism 4 could also be used to explain the effects of other anions on the activity of the enzyme.

Despite the great amount of work on effects of anions, no investigator has studied the effects of concentration of a wide range of anions, over a range of fumarate concentrations, under a single set of conditions. It is therefore impossible to detect from the literature some relationship between the structure and effect of the added anion (except for inhibition by substrate analogues, ref. 3).

5) Substrate activation: The initial rates of the fumarate hydratase reaction do not fit a simple Michaelis-Menten equation, except at low substrate concentrations (from zero to approximately 5 K_m's)(11). At higher concentrations, substrate activation is observed and at still higher concentrations, substrate inhibition is obtained in both directions. Massey (2) suggests that substrate activation is merely another case of anion effects on the enzyme activity, and could be explained by the same theory which he uses to explain the other anion effects (page 10). Alberty (1) too explains substrate activation with the same model that he used to explain the phosphate effect, i.e. fumarate combines at two sites on the enzyme molecule: the enzymic site and the activating site (Mechanism 4). Combination at the activating site has the same effect on the kinetics as does combination of phosphate at that site; the V_{max} for the substrate activated enzyme is the same as the V_{max} which is obtained in the presence of a very high concentration of phosphate. Alberty (1) derived Equation 5 on the basis of this mechanism and showed that the initial rate data can be fitted to this 2/1 function in the range of substrate concentrations where substrate inhibition does not occur:

$$v = \frac{V_2 + V_1K_2/(1 - V_1/V_2)(S)}{1 + K_2/(1 - V_1/V_2)(S) + K_1K_2/(1 - V_1/V_2)(S)^2} \qquad (5)$$

where K_1 and K_2 are constants of the Michaelis type, K_1 at low substrate concentrations and K_2 at high substrate concentrations. V_1 is the maximum initial velocity obtained at low substrate concentrations (from the 1/v intercept of the asymtote of the 2/1 function) and V_2 is the maximum initial velocity at high

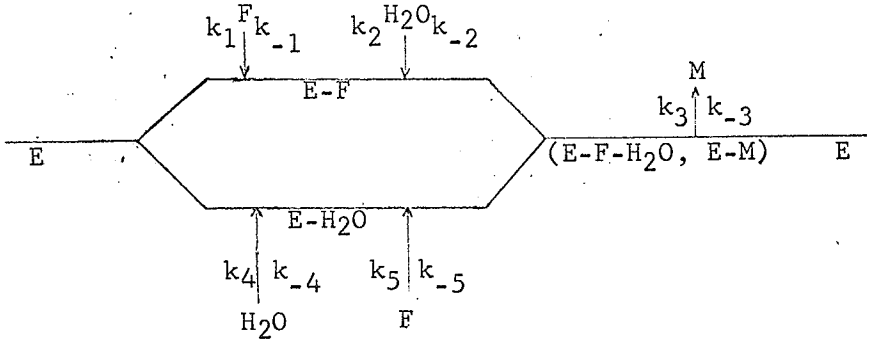
substrate concentrations. As the substrate concentration is reduced this equation simplifies to the usual Michaelis-Menten equation:

$$v = \frac{V_F\,(F)}{K_F + (F)} \quad \text{for fumarate, and } v = \frac{V_M\,(M)}{K_M + (M)} \quad \text{for L-malate} \qquad (6)$$

where the V's are maximum initial velocities and the K's are Michaelis constants; the subscripts F and M refer to the forward and reverse reactions respectively. (F) and (M) are fumarate and L-malate concentration respectively.

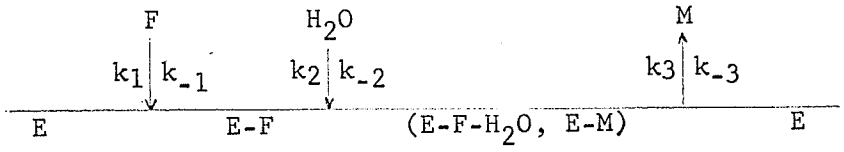
6) Mechanism: A bi-uni reaction such as the fumarase reaction may operate by one of three mechanisms (23):

1) Random bi-uni:



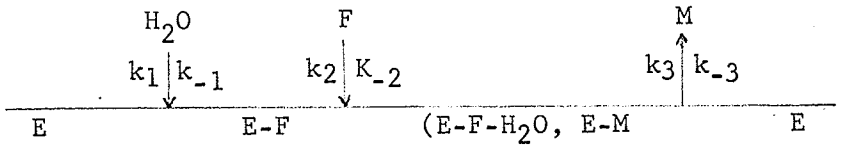
(7)

2) Ordered bi-uni, fumarate adding first:



(8)

3) Ordered bi-uni, water adding first:



(9)

where E is free enzyme, E-F and E-H_2O are transitory complexes

which participate in bimolecular reaction steps with a second substrate, and the parentheses indicate a central complex (cf. X_1---X_i---X_n in Alberty's mechanism, below).

For Mechanism 7, curved Lineweaver-Burk plots that are 2/1 functions are generally obtained except for special cases where one substrate is saturating, or when rapid equilibrium[1] obtains. The first of these cases is essentially the same as ordered bi-uni seen in Mechanisms 8 and 9. The rate equation for the rapid equilibrium random mechanism (see Appendix II) cannot be distinguished from ordinary sequential bi-uni rate equations by initial rate studies. The curved reciprocal plot obtained with fumarate cannot necessarily be taken to indicate that the reaction is random, since, as described above (page 13), it can be explained by assuming substrate activation. This assumption is supported by the facts that other anions activate and that a similar curved plot is obtained in the uni-bi direction with L-malate as substrate.

Alberty has therefore carried out most of his studies at low fumarate concentrations, in the range where the reciprocal plots are essentially linear. Under these conditions the kinetics

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[1] A rapid equilibrium mechanism is one in which the association and dissociation of substrates and products is many times faster than the interconversion of the central complex.

appear to obey the rate equation for a uni-uni mechanism of the type:

$$E + F \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X_1 \text{ --- } \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} X_i \underset{k_{-(i+1)}}{\overset{k_{i+1}}{\rightleftharpoons}} \text{ --- } X_n \underset{k_{-(n+1)}}{\overset{k_{n+1}}{\rightleftharpoons}} E + M \qquad (10)$$

The steady-state rate equation for the above mechanism has been derived (24):

$$-\frac{d(F)}{dt} = \frac{d(M)}{dt} = \frac{(V_F/K_F)(F) - (V_M/K_M)(M)}{1 + (F)/K_F + (M)/K_M} \qquad (11)$$

where K_F and K_M are Michaelis constants and V_F and V_M are the maximum initial velocities in the forward and reverse directions respectively. Alberty (25) has integrated Equation 11 and expanded the function as a power series in time (t). He showed that the reaction follows the integrated rate equation for a period comprising the first 25-30% of the approach to equilibrium in both the forward and reverse directions.

The Haldane relationship has been used by Alberty (1) to test the kinetic data for the fumarate hydratase reaction. The Haldane equation for the uni-uni mechanism is derived from rate equation 11 by setting v = 0 as at equilibrium:

$$K_{eq} = \frac{(M)}{(F)} = \frac{V_F K_M}{V_M K_F} \qquad (12)$$

Alberty (1) has found experimentally that equation 12 is obeyed over a wide range of pH and buffer concentration provided the kinetic constants are calculated from the linear portions of the Lineweaver-Burk plots at low substrate concentrations. Haldane equations for the bi-uni Mechanisms 7, 8 and 9 are given in Appendix II.

Although it is theoretically possible to distinguish between mechanisms 7, 8 and 9 on the basis of product inhibition studies (26, 27), such studies are hampered by the fact that one of the reactants (H_2O) is also the solvent for the reaction medium, and thus is always present at a fixed concentration.

b) Regulation and Allosteric Proteins:

At least two feedback systems control enzymic activity, one regulating the synthesis of the enzymes, another regulating their activity. The regulation of activity may occur by two processes, conversion from an inactive to an active form (activation), and inhibition or stimulation by signals in the cell. Only the effects of signals[2] on the activity of enzymes will be considered here.

Feedback inhibition occurs when the first enzyme of a sequence of reactions is inhibited by the end-product of that sequence. For example, aspartate transcarbamylase (ATCase) catalyses the first reaction unique to pyrimidine biosynthesis (28, 29). The product of this reaction is carbamyl aspartate, which is converted via six subsequent steps to the pyrimidine nucleotides, CTP and UTP. In Escherichia coli the rate of synthesis of pyrimidine nucleotides is regulated by one of the

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2 Signals: substrates, metabolites, or hormones that produce alterations in the kinetics of an enzyme by binding at specific sites on the enzyme molecule.

end-products, CTP. The basis of this regulation is the pronounced sensitivity of ATCase, the first enzyme of the pathway, to inhibition by CTP. Many pathways, in addition to the pyrimidine pathway, appear to be controlled by feedback inhibition (30) in bacteria and apparently also higher organisms (31).

The biological energy producing systems are regulated by the energy demand of the cell. The ATP regenerating systems of muscle, for example, increase their output of ATP at least 5-fold during exercise, and slow down again during rest. Considerable investigation of the regulation of the enzymes of glycolysis has been done and the adenine nucleotides (along with other metabolites) have been shown to play an important role. Since many of the glycolytic reactions also serve purposes other than the supply of energy from glucose, a complete analysis and correlation of the various signals is impossible at present. However, it has been demonstrated that ATP exerts an influence on glucose metabolism by inhibition at several steps of the glycolytic sequence of reactions. Thus, phosphorylase b, phosphofructokinase, aldolase, phosphoglycerate kinase, and pyruvate kinase are all inhibited by ATP. Of these, the first two enzymes are also known to be activated by 5'-AMP (32, 33). It appears that these enzyme possess highly specific binding sites for the regulatory molecules. These sites have been called allosteric sites.

Since allosteric enzymes are designed to obey very selective signals[2] in the cell, and thus are of great importance in regulation,

the properties of allosteric proteins will be discussed here. Allosteric proteins are assumed by Monod, Wyman, and Change ux (32) to be polymers, molecules composed of identical subunits, that have a definite axis of symetry. Regulatory changes in an allosteric molecule are conceived of as arising from a shifting back and forth between two states. The polymer can exist in a "relaxed" state or a "constrained" state. In one condition it binds substrate and activator; in the other state it binds inhibitor(s). The binding of such signals tilts the balance toward one or the other state but the molecule's symetry is preserved. The kinetics of a number of allosteric enzymes have been shown to be compatible with this model. The general properties of allosteric proteins are:

1) Most allosteric proteins are oligomers, involving identical subunits.

2) Allosteric interactions frequently appear to be correlated with alterations of the quaternary structure of the proteins (i.e. alteration of the bonding between subunits).

3) While heterotropic effects[3] may be either positive or negative (i.e. co-operative or antagonistic), homotropic effects appear to be always co-operative.

4) Few, if any, allosteric systems exhibiting only heterotropic effects are known. In other words, co-operative homotropic effects are almost invariably observed with at least one of the two (or more) ligands of the system.

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[3] Heterotropic effects: interactions between <u>different</u> ligands.
Homotropic effects: interations between <u>identical</u> ligands.

5) Conditions, or treatments, or mutations which alter the heterotropic interactions also simultaneously alter the homotropic interactions.

Aspartate transcarbamylase (ATCase, page 17) illustrates some of the properties of allosteric proteins. ATCase from E. coli (29) is a compact globular protein with a sedimentation coefficient ($S_{20,w}$) of 11.7 S, and a molecular weight of 3.1×10^5. Upon the addition of urea, or of the mercurial, p-mercuribenzoate, native ATCase dissociates into two types of subunits which are easily separable. The larger with $S_{20,w} = 5.8$ S and a molecular weight of 9.6×10^4, possesses the entire catalytic activity of the native enzyme and is completely insensitive to inhibition by CTP. The native enzyme contains two such catalytic subunits. The second protein, with $S_{20,w} = 2.8$ S and a molecular weight of 3×10^4, is enzymically inactive and is unnecessary for the activity of the catalytic subunit. This smaller protein, termed the regulatory subunit, bears the receptor sites for the inhibitor, CTP, and is required for the control of enzymic activity. There are four such subunits in each ATCase molecule. Upon mixing of the separated subunits from which the p-mercuribenzoate had been removed, aggregation occurs spontaneously to produce a complex having the catalytic and regulatory properties of the native enzyme. Desensitization[4] is not necessarily a

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[4] Desensitization, on the basis of this model, occurs when certain bonds break, which then prevent the conformational change from occuring.

property of all allosteric enzymes.

Substrate saturation curves, velocity vs. aspartate concentration, for ATCase are sigmoid shaped. As the CTP (the inhibitor) concentration is increased the curve becomes more sigmoid (28) and the half-saturation point is shifted to higher aspartate concentrations. The sigmoid shaped curves are explained by subunit interaction. A mathematical treatment based on the model previously described (page 18) yields qualitative agreement with the experimental data (28). The rate equation (13) is of the same form as the Hill formulation which assumes that the concentrations of intermediates between the relaxed and the constrained states of the protein are negligible.

$$v = \frac{V\,(S)^n}{K + (S)^n} \tag{13}$$

However this derivation assumes rapid equilibrium kinetics and is not valid when the combination of substrates with the enzyme is the rate-limiting step in the reaction.

The reciprocal plot for the above equation is given by Equation 14:

$$\frac{1}{v} = \frac{K}{V}(1/S)^n + \frac{1}{V} \tag{14}$$

where n is the number of (homotropic) interacting substrate binding sites on the enzyme molecule. It may be seen that Equation 14 describes a parabola for the case where n = 2. It should be pointed out, however, that a parabolic relation of this kind is also predicted by any model which assumes that the enzyme must attach to two substrate molecules for reaction to occur

(i.e. ES is inactive, while ES_2 is active and reacts to give products + ES). Thus, when such a substrate is the variable substrate it gives parabolas if the two points of addition are reversibly connected in the reaction sequence (or higher degree functions if there are more than two points of addition reversibly connected) (ref. 27).

The activation and inhibition of ATCase by maleate is very interesting because the effect is similar to that shown by phosphate on fumarate hydratase activity. Maleate, a substrate analogue, activates ATCase at low concentrations and inhibits at high concentrations. The activation occurs because maleate binds at some of the substrate binding sites and helps to stabilize the active form of the enzyme. This is similar to the activation produced by the substrate (homotropic effects) but differs from the activation produced by heterotropic activators in that there is no site specific for maleate on the enzyme. Increasing the maleate concentration above the concentration required for optimum stimulation results in an inhibition of the enzyme. The inhibition is of the ordinary competitive type and is due to the displacement of aspartate from the substrate binding sites. The heat desensitized enzyme shows no activation by maleate, only competitive inhibition.

Monod et al (32) do not treat the case where both conformations of the allosteric enzyme are active. This situation could be described as partial allosterism to differentiate it from total allosterism, described above. In partial allosterism the K_m and V_{max} are modified by the conformational changes in

the enzyme which occur when the substrate or other 'effector' (either activator or inhibitor) binds at the 'allosteric' site. The rate equation for such a situation has been derived by Botts and Morales (34) and is given in Appendix II.

C. EXPERIMENTAL

Two preparations of fumarate hydratase were used in these experiments. The experimental section is therefore divided into two parts: the first dealing with the partly purified yeast preparation and the second with the crystalline pig heart enzyme.

I. YEAST FUMARATE HYDRATASE

Methods

a) Preparation of Fumarate Hydratase:

Fumarate hydratase from bakers' yeast was prepared as follows. One-third kilogram of air-dried yeast was suspended in a liter of 0.1 M sodium bicarbonate and allowed to autolyse with stirring for eight hours at 37° C. The solid material was removed by centrifugation at 12000 × g for 20 minutes. To the supernate was added 36 grams ammonium sulfate per 100 ml (supernate) and the mixture was stirred for 20 minutes at room temperature. After centrifugation the supernatant fluid was discarded and the pellet dissolved in 100 ml cold deionized water[5]. To this solution was added 23 gm ammonium sulfate per 100 ml of volume before addition of the salt and the mixture stirred and centrifuged as above. To the supernatant fluid[6] was added 5 gm ammonium sulfate per 100 ml. The mixture was stirred for 20 minutes in the cold, centrifuged at 15000 × g for 20 minutes, and the precipitated protein was dissolved in water (approximately 100 mg protein per ml).

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[5] The water used throughout this study was first distilled and then deionized by passing it through a Barnstead mixed resin cartridge (Canlab 30-867).

[6] The supernate was a gift from William Bridger who carried out the procedure to this point in the preparation of adenylosuccinase from yeast (35).

The partially purified enzyme was dialysed against two changes of 15 mM EDTA, 10 mM tris acetate pH 7.0 at 0° C for a total of 7 hours. The dialysed enzyme was either frozen directly, or first diluted with 10 or 100 volumes of 15 mM EDTA and 10 mM tris acetate and then stored frozen at -20° C. Changes from this procedure, such as the omission of the dialysis step or of EDTA, had no apparent effect on the kinetics of the enzymic reaction.

The purification from the crude extract stage was six-fold and the preparation possessed some adenylosuccinase activity.

b) Assay of Fumarate Hydratase Activity:

The spectrophotometric method (36), which depends on the UV absorbtion of the double bond of fumaric acid, was used to determine the enzyme activity. The fluorescent method of Speck (37) was also attempted but proved less convenient and less reliable than the spectrophotometric assay.

The procedure adopted for the assay consisted of mixing the reactants in the cuvette and reading changes in absorbance as the reaction proceeded. In order to study the kinetics over a wide range of substrate concentration the sensitivity of the method was varied. Since this required the use of cuvettes with different light paths, the volume of the assay medium and the amount of enzyme used were varied (0.5 ml for 2 mm light path, 0.5 or 1 ml for 10 mm light path, and 3 ml for 50 mm light path). All stock solutions were made up at 10 or 20 times the

final concentration and adjusted to pH 7.0 with 1 N KOH. The volumes of the components were usually in the ratio indicated in the following example:

	Volume	% of final volume	Final concentration
Buffer (12.5 mM tris acetate)	400 µl	80%	10 mM
Substrate solution	25 µl	5%	variable
Compound tested	25 µl	5%	variable
Water	40 µl	8%	
Enzyme solution	10 µl	2%	
Total	500 µl	100%	

When substrate concentration was varied or when additional compounds were added, the total volume was kept constant by changing the amount of water added. The enzyme was always the last addition, after which rapid mixing was effected with several strokes of a close-fitting plastic stirrer. The absorbance was recorded on a Cary 15 recording spectrophotometer at chart speeds of 10 seconds/division to 250 seconds/division (1 division = 1/3 inch), depending on the reaction rate. The wave-length chosen varied from 220 mµ to 305 mµ depending on the substrate concentration used, and the rates were corrected according to the difference spectrum for the fumarate hydratase reaction given by Alberty (1). The cell compartment was kept at 25° C, as was the buffer. The other solutions were at room temperature (25 $\pm$ 2° C), and the enzyme was kept on ice.

Because of the diverse effects of anions on the kinetics of pig heart fumarate hydratase, Alberty has used tris acetate

buffer for many of the kinetic studies. Since it is possible to maintain a constant acetate ion concentration over a wide range of pH, the use of this buffer obviates the variation in anionic composition with pH that occurs with phosphate buffer (11). A final concentration of tris acetate of 10 mM was used throughout this study since acetate appears to have only a slight stimulatory effect at this concentration. The exact degree of stimulation is unknown because no assays were done in the absence of buffer. It is known that this stimulation is far from the maximal stimulation which acetate ion may produce. For this reason, 10 mM tris acetate buffer is not likely to mask any of the stimulatory effects of other anions as happens when high buffer concentrations are used. The buffering capacity of tris acetate at pH 7 is low because the pK^{1}s of tris and acetic acid are 8.15 and 4.75 respectively. In order to avoid overtaxing the buffer, therefore, all solutions used in the assay were adjusted to pH 7.0.

A further source of difficulty is the large temperature coefficient of the pH of tris buffers (0.3 pH units/degree at 25° C). Since in early experiments the buffer was adjusted to pH 7 at room temperature, the pH from experiment to experiment may vary $\pm$ 0.06 pH units. However, this variation was found not to affect the reaction rate sufficiently to alter the interpretation of the results.

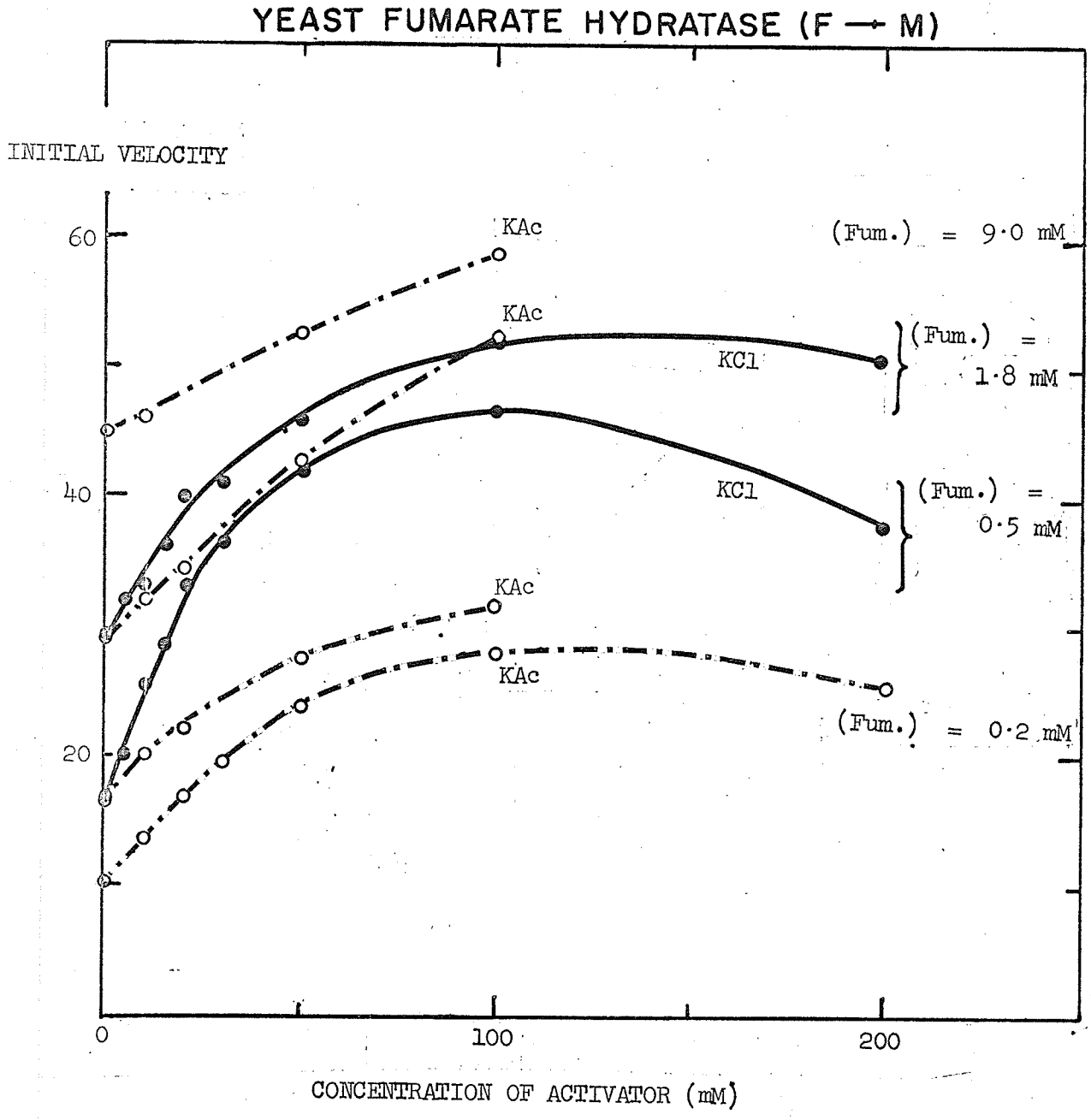

Figure 1. Activation by chloride and acetate. These results are taken from three separate experiments and have been corrected to the same control values. The assay medium contained 0.15 mM EDTA and 10 mM tris acetate pH 7.

Results

Certain references to the literature will be made in this section for comparison with the results reported in this thesis. Where this is the case, the conditions of the assay and the source of the enzyme are cited.

c) The Effects of Ions on Fumarate Hydratase Activity:

i) Effects of chloride and acetate: Effects of anions on the kinetics of pig heart fumarate hydratase have been described in the Literature Review. The present study shows that anions also affect the yeast enzyme. The effects of a variety of salts were studied, and Figure 1 shows the effects of KCl and K acetate on the rate in the direction fumarate to malate. Both compounds stimulate the enzyme with maximal stimulation between 100 and 200 mM. Inhibition of the enzyme by high concentrations of chloride and acetate occurs at low fumarate concentrations. The difference observed between the effects of chloride and acetate are significant and show that two univalent anions stimulate fumarate hydratase to a different extent.

ii) The effect of cations: It is known that the substitution of potassium and ammonium ions for sodium ions is without effect on the kinetics of pig heart fumarate hydratase (38). The non-specificity toward univalent cations was confirmed by testing NaCl, KCl, and CsCl at 100 mM concentration (Table III, expt. 1, and Figure 2).

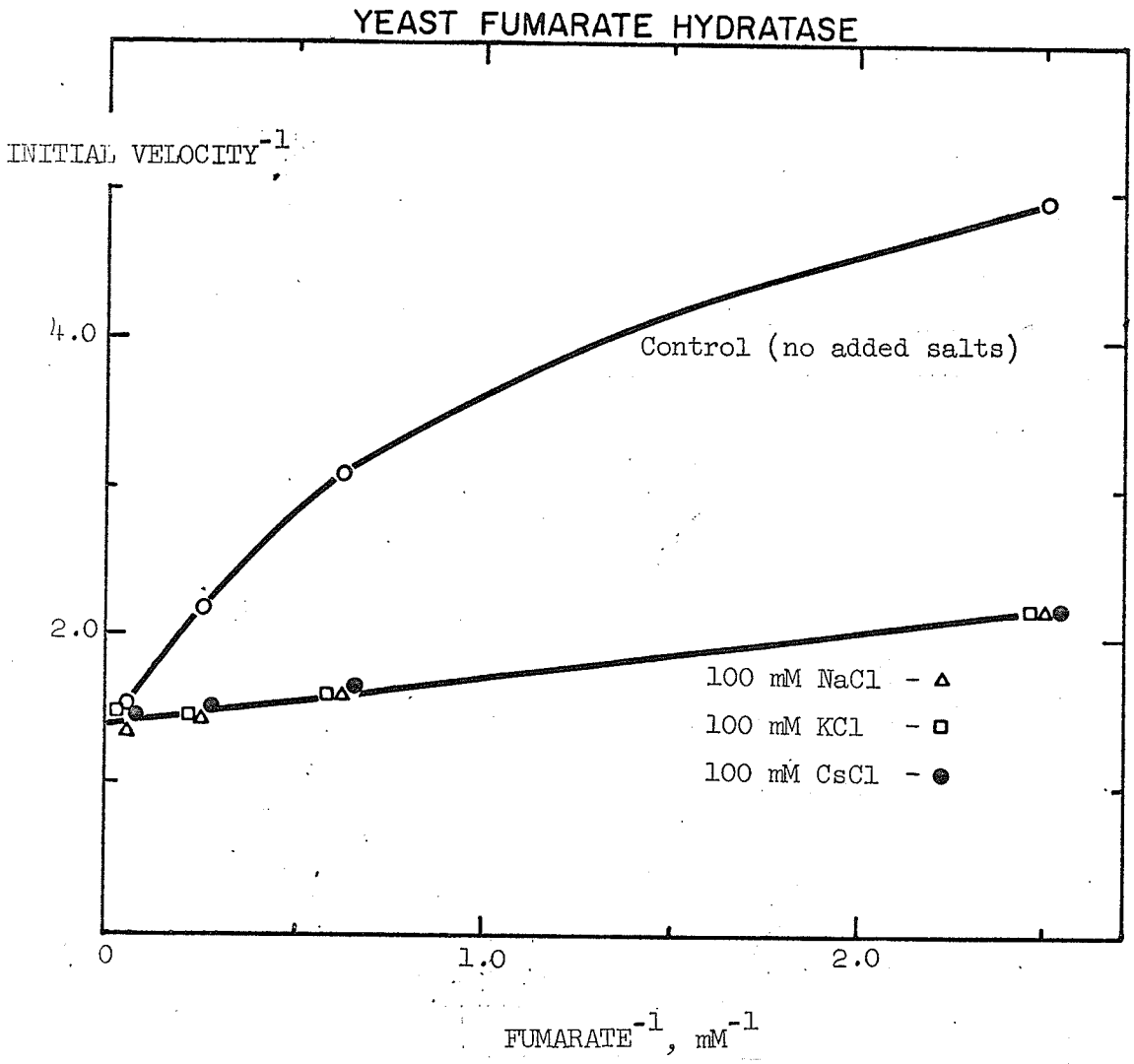

Figure 2. Lineweaver-Burk plot showing activation by 100 mM chloride ion. Points for KCl and CsCl are slightly displaced from the true fumarate concentration so that all three points may be seen. The assay medium contained 0.2 mM EDTA, 2 mM phosphate and 9 mM tris acetate pH 7.

$Ca(acetate)_2$ and $Mg(acetate)_2$ were compared with K acetate and were found to give almost equal activation at equal concentrations up to 30 mM acetate (Table III, expt. 2). Inhibition is seen at higher magnesium and calcium concentrations (Table III, expt. 3 and 4) when compared to potassium acetate, but the inhibition is small except at very high concentrations of the divalent cations. It is concluded from these experiments that the stimulatory effects of salts is determined by the nature of the anion.

iii) Effect of fumarate: Since anions stimulate the enzyme it would be expected that fumarate should also stimulate, and in fact fumarate has been shown to stimulate the pig heart enzyme (1). Figure 2 shows the effects of fumarate concentration on the yeast enzyme. Initial velocities were determined at four fumarate concentrations in the presence and absence of 100 mM chloride. In the absence of added salts the resulting reciprocal plot is biphasic due to substrate activation at high fumarate concentrations, as found for the pig heart enzyme by Alberty (1). Sodium, potassium, or cesium chloride all have the same effect, activating the enzyme and abolishing the biphasic nature of the reciprocal plot. The maximum initial velocity, indicated by the y-intercept, is unchanged by the presence of 100 mM chloride. Since fumarate does not activate at high salt concentrations and also since high fumarate concentration produces the same V_{max} as high salt concentration it appears probable that the mechanism of the stimulation by fumarate is similar to that for

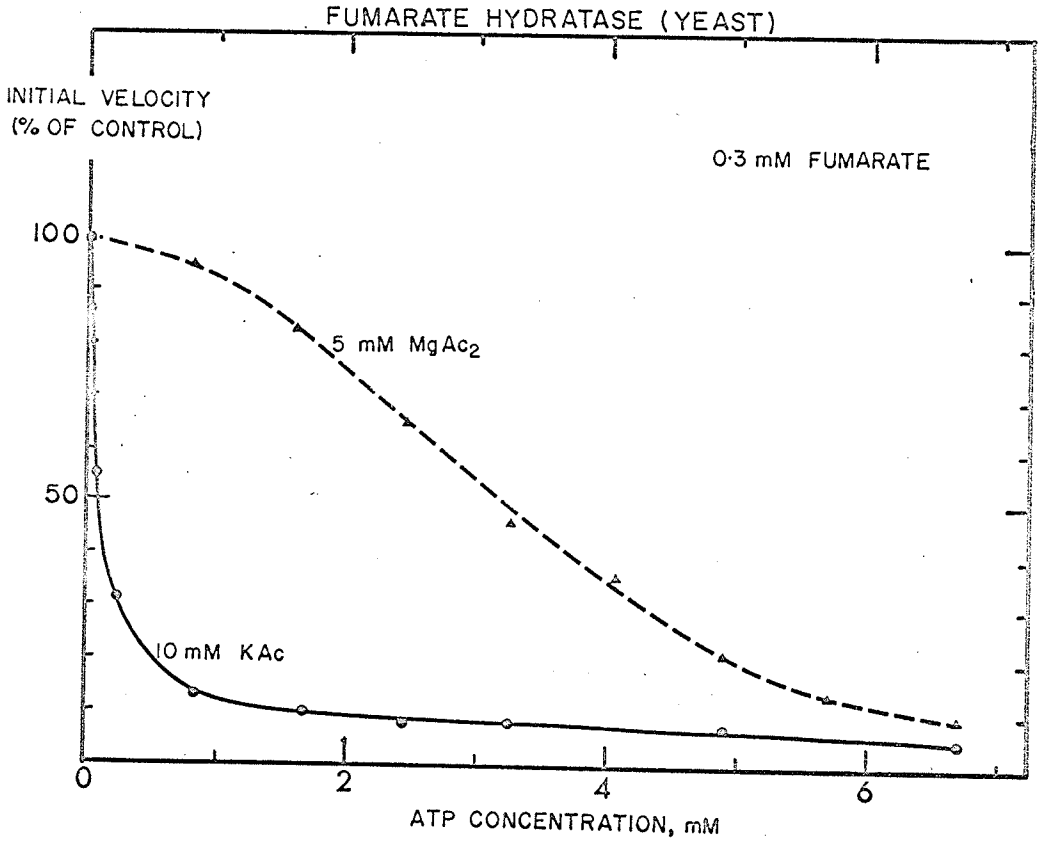

Figure 10. The ATP inhibition of fumarate hydratase in the presence of 5 mM Mg^{++}.

TABLE III

Activation of Fumarate Hydratase by Sodium, Potassium, Cesium, Calcium, and Magnesium Salts (Yeast Enzyme, M — F)

Activator	Activity (% of Control)		
	4.0 mM Fumarate	0.4 mM Fumarate	0.2 mM Fumarate
Expt. 1			
100 mM NaCl	151	226	
100 mM KCl	148	226	
100 mM CsCl	141	222	
Expt. 2			
30 mM K acetate			192
15 mM $Mg(acetate)_2$			191
15 mM $Ca(acetate)_2$			203
Expt. 3			
100 mM K acetate			270
50 mM $Mg(acetate)_2$			235
50 mM $Ca(acetate)_2$			219
Expt. 4			
200 mM K acetate			246
100 mM $Mg(acetate)_2$			175
100 mM $Ca(acetate)_2$			164

The Control assay contains 10 mM tris acetate in expts. 2, 3 and 4. Expt. 1 contains 2 mM phosphate in addition to 9 mM tris acetate.

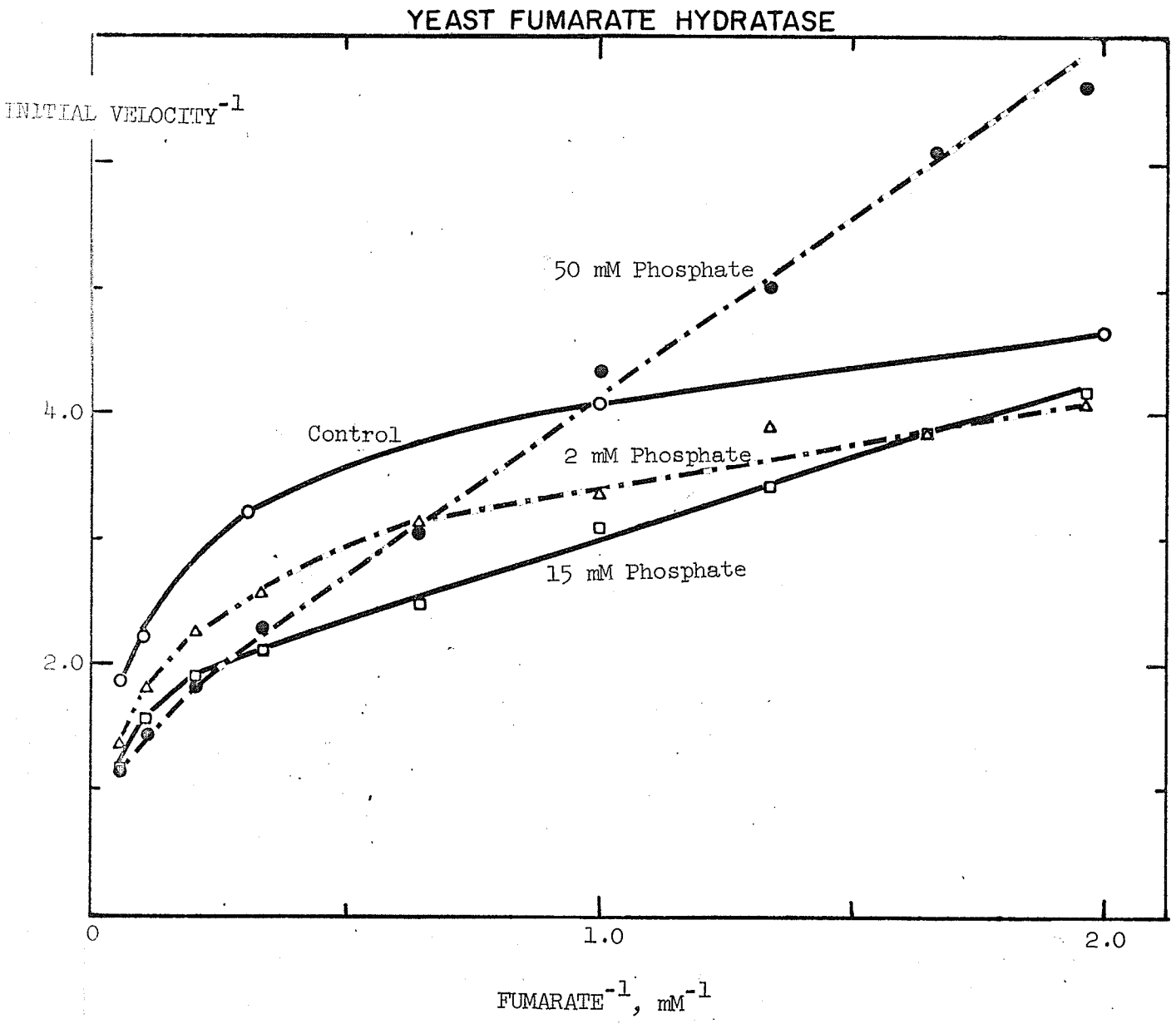

Figure 3. Lineweaver-Burk plot showing the effect of inorganic phosphate on the activity of fumarate hydratase in the forward direction.

chloride. One of the effects of these anions is clearly an increase in V_{max}. Potassium chloride also increases the K_m. The K_m calculated for the lower line (100 mM KCl present is 0.23 mM. The fumarate concentration was not low enough in this experiment to obtain an accurate value for the K_m for the upper line (i.e. KCl absent). However, a subsequent experiment at lower fumarate concentrations yielded a value of 0.042 mM (Fig. 6 Control).

iv) Anions with a charge of -2: The effects of phosphate upon the kinetics of pig heart fumarate hydratase have been studied (1, 21) and a reaction mechanism proposed. The effect of phosphate on the yeast enzyme is shown in Figure 3, plotted as reciprocals. The curved lines indicate substrate activation, and at high phosphate concentration (50 mM) an almost linear plot is obtained as was the case with chloride. The intersection point to the right of the y-axis indicates that there is activation by phosphate at high fumarate concentration and inhibition at low fumarate concentration. This crossover point depends on the phosphate concentration, i.e. at high phosphate concentration the inhibition is greater than at low phosphate concentration and the crossover occurs at higher fumarate concentration. This is further illustrated by the results described in the following paragraph.

Experiments were done with a variety of anions to determine the specificity of the ionic effect. In these experiments the salt was increased in concentration at a fixed fumarate

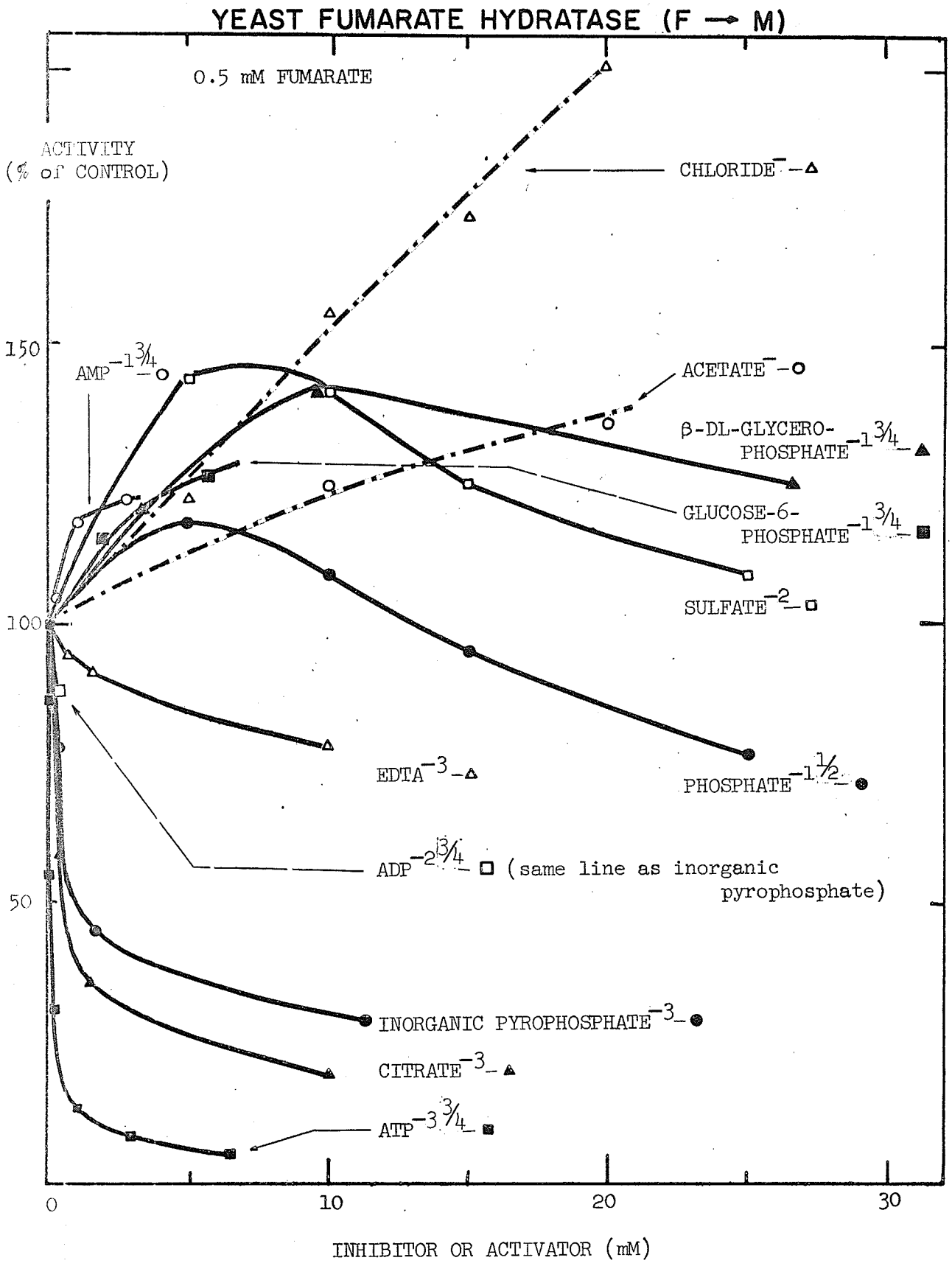

Figure 4. The effect of anions on the activity of yeast fumarate hydratase.

TABLE IV

Sulfate and Phosphate Effects on Fumarate Hydratase Activity

(Yeast Enzyme, F — M)

	Relative reaction rates		
	0.5 mM Fumarate	1.8 mM Fumarate	9.0 mM Fumarate
Sulfate concentration			
0	36	49	68
10 mM	51	59	78
25 mM	39	58	76
50 mM	30	51	79
Phosphate concentration			
0	36	48	68
10 mM	39	60	(90)
25 mM	28	58	(94)
50 mM	17	48	99

The values in parentheses were obtained from another experiment (data shown in Figure 3) and are corrected to the same control value.

concentration as seen in Figure 4. Sulfate, like phosphate, stimulates at low concentrations and inhibits at high concentrations. At all fumarate concentrations phosphate was similar to sulfate; however, at low fumarate concentrations the inhibition appeared at lower salt concentrations, and at high fumarate (9 mM) the phosphate and sulfate only activated up to 50 mM anion concentration (Figure 3 and Table IV). It can also be seen (Figure 4) that an organic phosphate, β-glycerophosphate, is quite similar to inorganic phosphate in its effects on fumarate hydratase. Glucose-6-phosphate, which has been shown to be important in the regulation of glycolysis at the phosphofructokinase step was also tested and found to have the same effect as β-glycerophosphate up to 5.8 mM concentration (Figure 4). The organic phosphates thus do not appear to be of direct importance in the regulation of fumarate hydratase activity.

v) Anions with a charge greater than -2: The other anions that were tested are all metabolites except EDTA, which was included for comparison with the trivalent anions, ADP, PP_i, and citrate. All of these inhibited and no sign of stimulation could be seen at any of the concentrations tested.

The effects of the anions appear to fall into three classes. The singly charged anions (chloride and acetate) only activate except at very high concentrations. The doubly charged anions activate at low concentrations and inhibit at

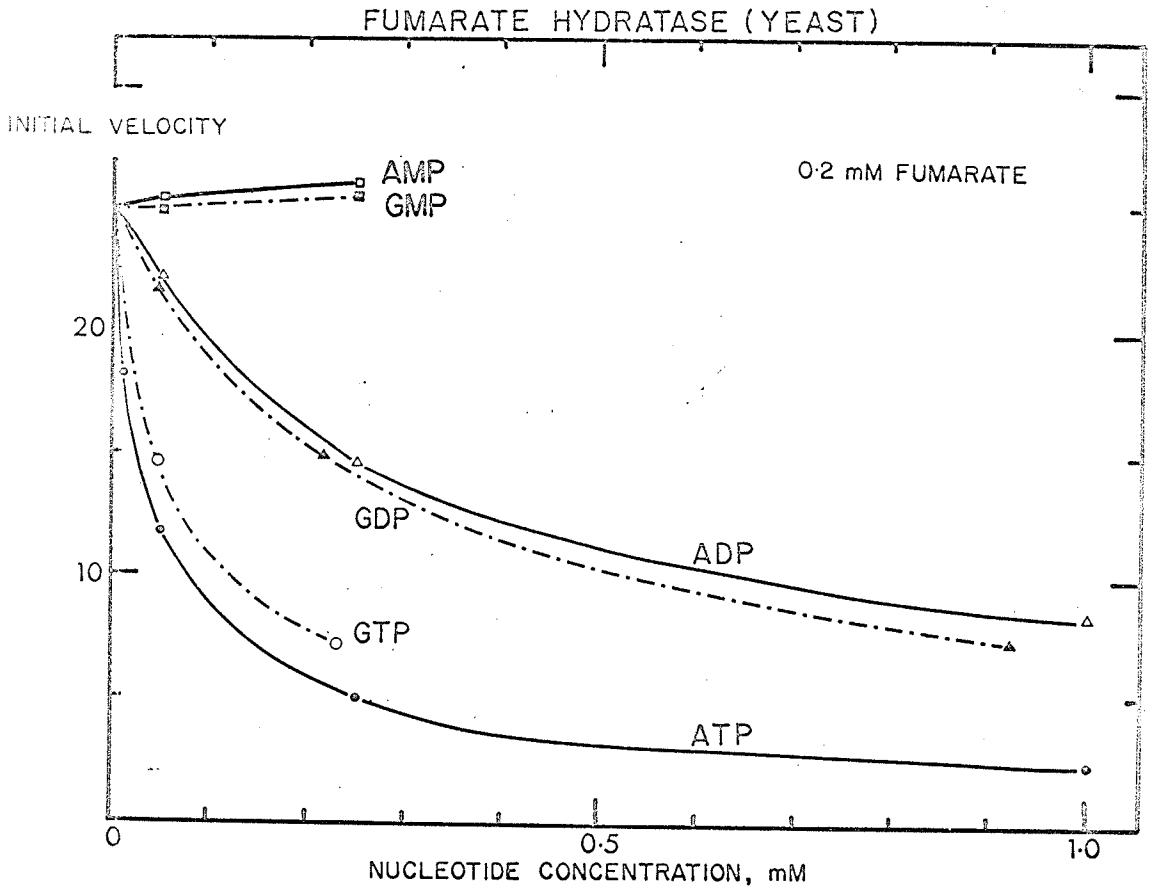

Figure 5. The effect of the nucleoside mono-, di-, and triphosphates on the activity of fumarate hydratase.

high concentrations. Anions with a charge greater than -2 only inhibit the enzyme under the conditions used in these experiments. However, the large difference between EDTA and citrate, both triply charged anions, shows that the degree of inhibition is not related solely to the ionic charge, but also to the molecular structure.

d) The Effect of Nucleotides:

i) The mono-, di-, and triphosphates of adenosine and guanosine: The effects of nucleotides on fumarate hydratase activity were studied because of their importance in metabolism and energy supply in biological systems. Special experimental difficulties arose because of the UV absorbtion spectra of the nucleotides; consequently, only low concentrations could be used. In order to keep the total optical density below 3, activity measurements were made at wavelengths where the absorbencies of adenosine and guanosine are minimal (225-230 mμ and 290-300 mμ).

The effects of ATP, ADP, and AMP are shown in Figure 4 for comparison with the other anions. Figure 5, on an expanded concentration scale, shows ADP to be about 1/10 as potent an inhibitor as ATP[7]. It should be noted that this ADP contained about 3% ATP but that similar results were obtained when purified ADP (see Appendix I) was used. AMP did not inhibit the enzyme at all. The results with AMP were obtained at the

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[7] Ten times as much ADP is needed to produce the same degree of inhibition that a given concentration of ATP produces.

isoabsorbtive point of the adenylosuccinase reaction[8]. The effects of the guanine nucleotides are similar to the adenine nucleotides, although the purity of the guanine nucleotides was not determined.

ii) A comparison of the nucleoside triphosphates and AMPS: In order to determine whether the effect of ATP was simply due to the four negative charges on the anion, ATP was compared with the pyrimidine nucleoside triphosphates and with adenylosuccinate (which does not have the triphosphate group, but has four negative charges at pH 7) (Table V). It was not possible to use enough AMPS to obtain a substantial degree of inhibition because readings could only be made at 259.5 mμ, which is the isoabsorbtive point for the adenylosuccinase reaction[8]. The use of cuvettes with 2 mm light paths permitted the use of 0.4 mM AMPS, although it also diminished the sensitivity and accuracy of the activity measurement. In Table V are seen the concentrations of inhibitor required to produce 25% and 50% inhibition. It can be seen that ATP is almost twice as effective as CTP, UTP, or GTP, and 10 times as effective as AMPS, even though all of these compounds have

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8 As mentioned earlier, the enzyme preparation contained a detectable amount of adenylosuccinase. When reactions were carried out in the presence of AMPS or AMP and fumarate, it was possible to eliminate interference by the adenylosuccinase reaction by carrying out the measurement of fumarate hydratase activity at 259.5 mμ, a wave-length where the adenylosuccinase reaction causes no change in absorbance.

similar ionic charges at the pH of the experiment. The purity of ATP was checked and found to be at least 99%[9]. The purity of the other nucleotide triphosphates was not determined.

e) A Study of the ATP Inhibition:

i) Reciprocal plots: The effect of ATP at five concentrations (200-fold range) over a 300-fold range of fumarate concentrations is shown in Figure 6, plotted by the Lineweaver-Burk method. The following points should be mentioned:

1) the line drawn for the control is a computer fit to the 2/1 function (see equation 5) with the following kinetic constants: $V_1 = 27.7$, $V_2 = 99.0$, $K_1 = 0.042$ mM, $K_2 = 4.3$ mM; the standard error for these constants is about 75% of their value, except for V_2 which has a 5% standard error; the plots with ATP present do not fit the 2/1 function.

2) the lines are all non-linear, i.e. simple Michaelis kinetics do not apply, although at low fumarate concentrations the lines approach linearity.

3) the V_{max} is unchanged even at the high ATP concentration of 2 mM, indicating competitive inhibition.

4) the slope of the reciprocal plot is not a linear function of ATP concentration (see replot of slopes, Figure 8), i.e. saturating levels of ATP may not give complete inhibition.

ii) Inhibition of the reverse reaction by ATP: The initial rates were obtained for three malate concentrations and two ATP concentrations. The reciprocal plots, shown in Figure 7, are almost linear. However, there is an activation

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9 ATP was assayed with hexokinase and glucose-6-phosphate dehydrogenase, and the reaction followed to completion by the optical density increase at 340 mμ. More than 99% of the purine derivative present was ATP.

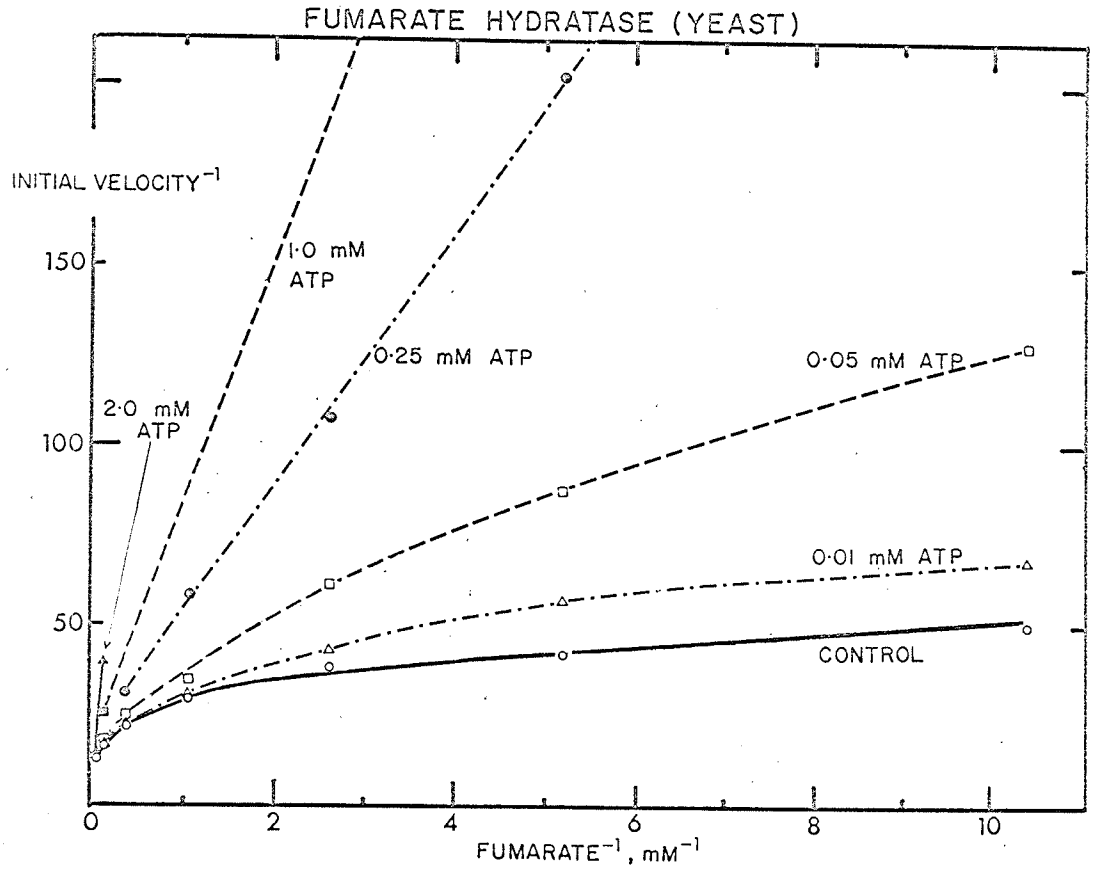

Figure 6. The inhibition of fumarate hydratase by ATP. The incomplete lines have additional points which are off the scale as drawn. The line drawn for the control is the computer fit to a 2/1 function.

TABLE V

Inhibition of Fumarate Hydratase by Nucleoside Triphosphates and AMPS (Yeast Enzyme, F M)

Inhibitor	Fumarate concentration	Concentration of Inhibitor (mM)	
		50% inhibition	25% inhibition
ATP	0.2 mM	0.043	0.012
GTP	0.2 mM	0.072	0.02
ATP	0.3 mM	0.078	0.02
CTP	0.3 mM	0.16	0.04
UTP	0.3 mM	0.13	0.03
ATP	0.5 mM	0.014	0.035
AMPS	0.5 mM	---	0.40

at high malate concentration but it is not easily seen with the intercept so near the origin as in Figure 7. The replot of slopes as described in e(iii) shows a concave downward curve (Figure 8). The K_i obtained from the x-intercept (low ATP) is approximately 1.5×10^{-5} M, close to the K_i found for the forward reaction (below). The K_m for malate as substrate is approximately 5×10^{-5} M.

iii) The replot of slope vs. ATP: Several graphical methods are available for the determination of inhibitor constants (10). In the Lineweaver-Burk plot, the slope of the reciprocal plot for simple competitive inhibition is given by $K_m(1 + I/K_i)/V_{max}$. A replot of the slope against I is a straight line:

$$\text{slope} = \frac{K_m}{V_{max}} + \frac{K_m}{V_{max}K_i}(I) \qquad (15)$$

The x-intercept of this line (i.e. at slope = 0) is equal to $-K_i$. In Figure 8 the replot of slopes at low fumarate concentration is shown for both the forward and reverse reactions of yeast fumarate hydratase. The curves are concave downward, suggesting either a partial competitive type of inhibition (type Ib described by Dixon and Webb, ref. 15, p. 320) or that ATP binds two forms of the enzyme which are connected by reversible steps in the reaction sequence, activating at one site and inhibiting at the other site (27). The K_i[10] calculated

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[10] Since the reciprocal plots are non-linear and the inhibition is not simple competitive, the K_i is here defined as the concentration of ATP that results in 50% inhibition at low fumarate concentration. The K_i was obtained by extrapolating the replot of slopes at low fumarate concentration to slope = 0 (the x-intercept, Figure 8). This is done to give some estimate of the potency of ATP as an inhibitor.

from the x-intercept at low fumarate and low ATP concentration is approximately 1.3×10^{-5} M. Other methods of plotting the data, described by Dixon and Webb (ref. 15, pp. 327-331), also yield only approximate values, 2.0×10^{-5} M (39) and 1.2×10^{-5} M (40), at fumarate concentrations of 0.1 to 0.4 mM.

iv) The effects of other anions on the ATP inhibition: The effect of several compounds on the inhibition by ATP was studied. These included certain compounds known to influence regulatory effects of ATP on other enzymes. The effects of P_i, AMP, KCl, and K acetate on the inhibition of fumarate hydratase by ATP are shown in Table VI. The data for AMP were obtained at 225 mμ and 230 mμ, at which wave-lengths the adenylosuccinase reaction contributes slightly to the measured rate. For this reason AMP was included in the controls without ATP, where 0.5 mM AMP was found to have only a small effect on the activity (6-8%, including actual stimulation plus adenylosuccinase activity). The data given in Table VI, expt. 2 show that no reversal of inhibition is obtained with AMP, even when AMP is in 10-fold excess over ATP. Partial attenuation of the inhibition is obtained with high concentrations of inorganic salts (Table VI, expt. 1, 3, and 4). This decrease in potency appears to result from a non-specific anion effect since chloride and phosphate both exhibit similar effects. The K_i for ATP in the presence of 50 mM KCl at the fumarate concentration of 0.2 mM may be calculated by Dixon's method (41), and turns out to be approximately 0.06 mM, which

is about 5 times higher than the K_i calculated by the same method in the absence of added salts (see above). It may also be seen from Table VI, expt. 3 that the increase in the concentration of ATP needed to bring about 50% inhibition of the enzyme in the presence of 50 mM KCl is approximately 5-fold. This is closely correlated with the increase in the calculated value of K_i.

f) Effect of Divalent Cations upon the Inhibition by Multivalent Anions:

i) Effect of Ca^{++} and Mg^{++} in the assay medium: Since in the cell a fraction of the nucleotide polyphosphates (or citrate) must be present as magnesium and calcium complexes, it is pertinent to know whether such complexes are inhibitors of the enzyme. Calcium and magnesium acetate have been shown, in an earlier section, to have little effect on the enzyme activity up to 15 mM concentration (Table III). A more detailed study, however, revealed a small activation of about 10% at low Mg^{++} and Ca^{++} concentrations (1 to 2 mM) that virtually disappeared at higher concentrations. Measurement of the pH, before and after adding enzyme to the Mg^{++} solution in 10 mM tris acetate buffer, showed a drop in pH of 0.1 unit, which accounts for at least one-half the activation seen[11]. This change in pH, corresponding to the titration of 0.14 mM of

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[11] A decrease in pH results in a slight activation of the enzyme because pH 7 is on the alkaline side of the pH optimum for the fumarate hydratase reaction (forward direction).

TABLE VI

Effect of Anions on the Inhibition of Fumarate Hydratase by ATP (Yeast Enzyme, F → M)

Anion	Fumarate conc. (mM)	ATP conc. at 50% inhibition (mM)	
		without anion	with added anion
expt. 1			
1 mM P_i	0.2	0.055	0.065
3 mM P_i	0.2	0.055	0.085
9 mM P_i	0.2	0.055	0.210
expt. 2			
0.5 mM AMP	0.1	0.030	0.028
0.5 mM AMP	0.4	0.065	0.067
expt. 3			
50 mM KCl	0.2	0.055	0.250
expt. 4			
6 mM K acetate	0.2	0.055	0.066

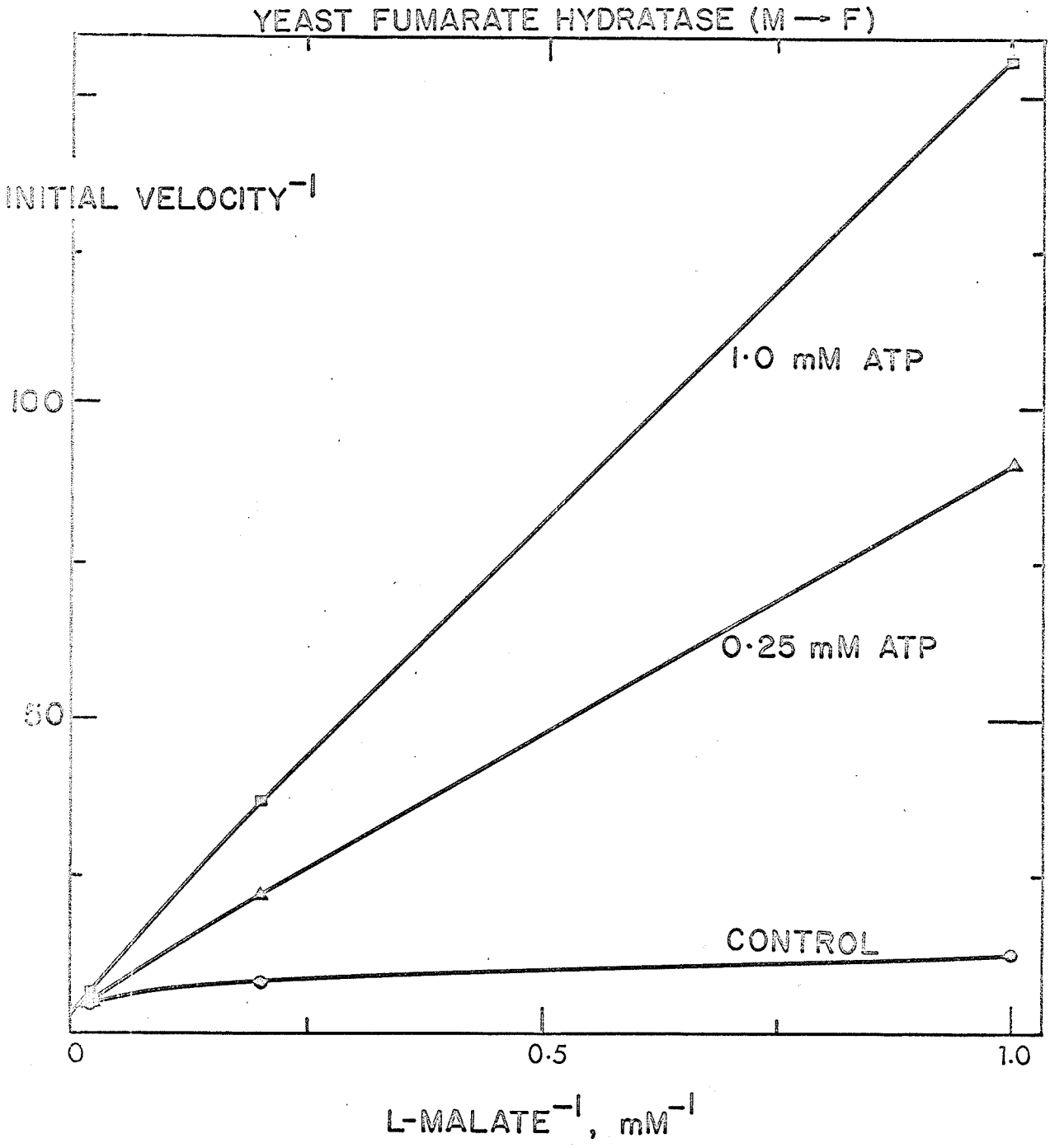

Figure 7. Reciprocal plot showing inhibition of fumarate hydratase by ATP.

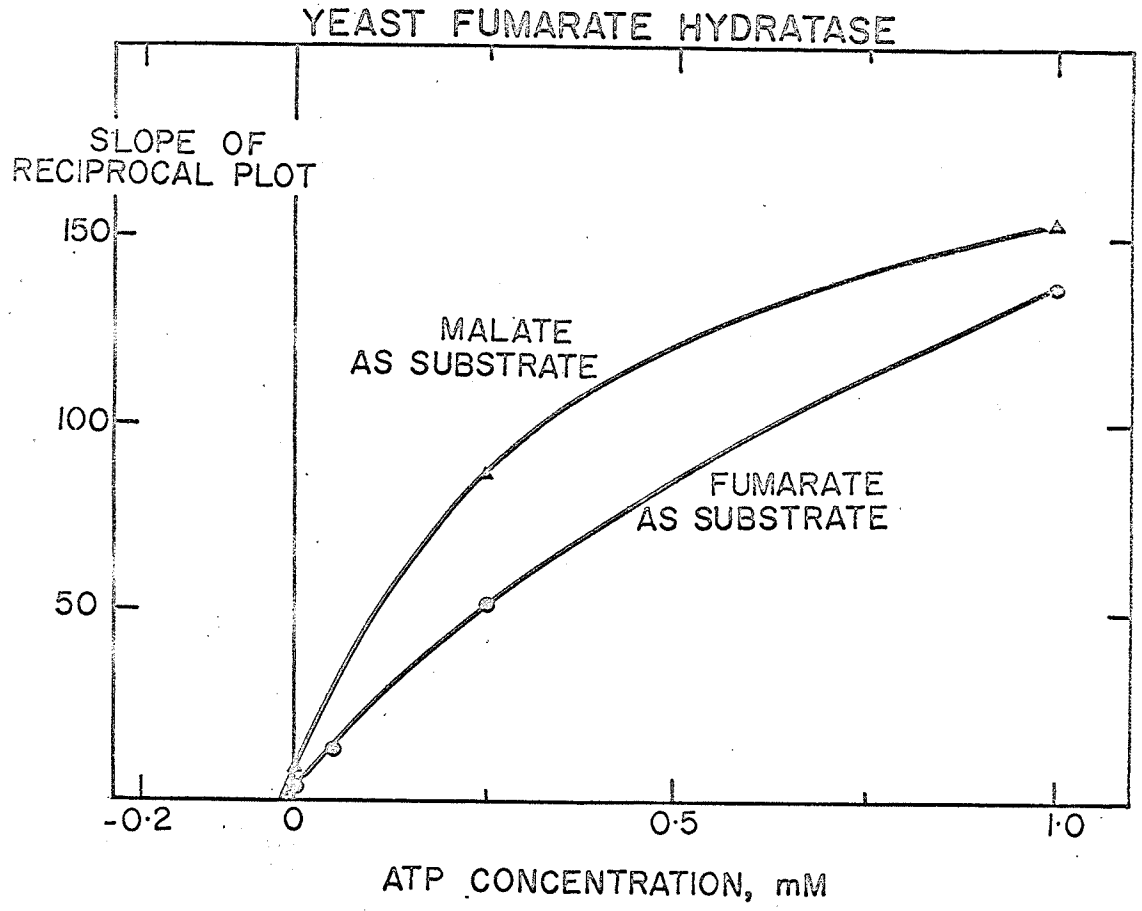

Figure 8. Replot of slopes of the reciprocal plots obtained for the inhibition of fumarate hydratase by ATP. The data is taken from Figures 6 and 7. The experiments in the forward and reverse directions were done with different enzyme concentrations.

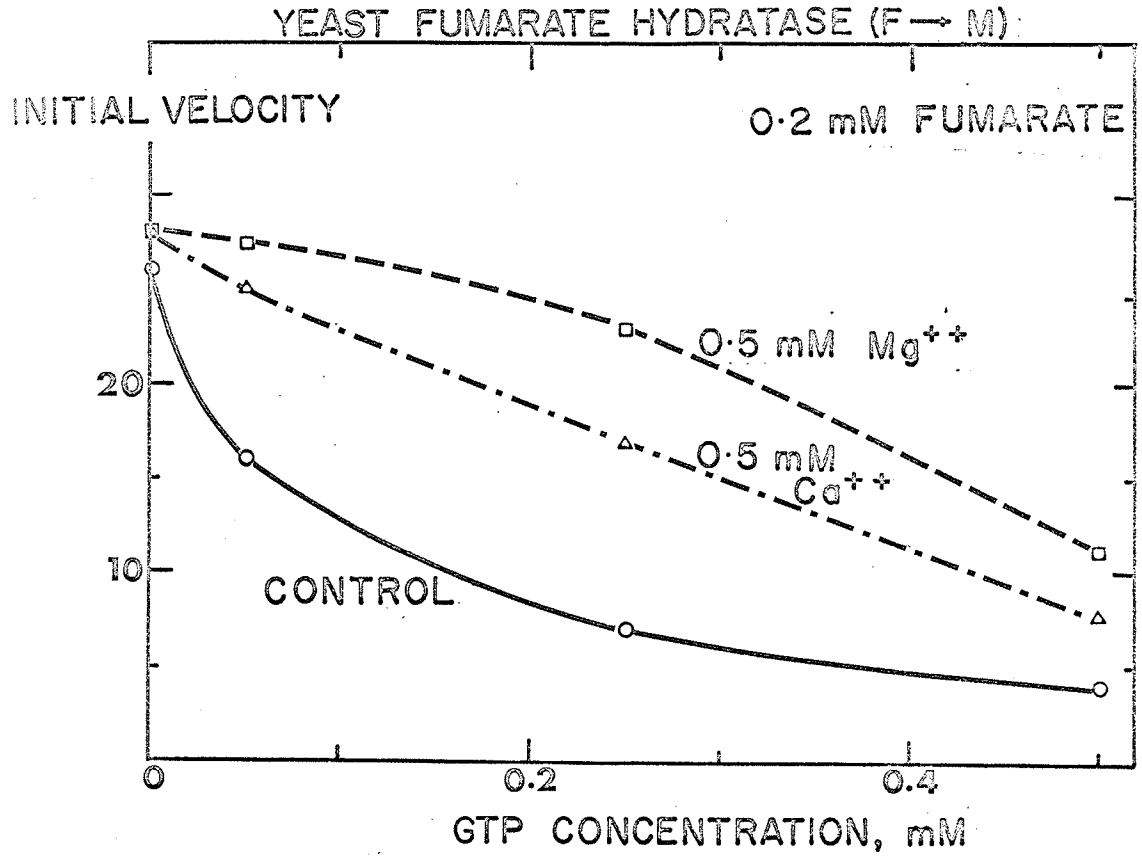

Figure 9. The effects of Mg^{++} and Ca^{++} on the inhibition of fumarate hydratase by GTP.

the 10 mM tris acetate buffer, is thought to be due to the release of protons resulting from the binding of Mg^{++} to either protein or EDTA. The 5% error due to pH changes will not affect the validity of the conclusions drawn from the experiments in this section of the thesis.

ii) Evidence that $MgATP^{=}$ does not inhibit: It is known that the binding constant for Mg^{++} and ATP^{-4} is in the range of 10^4 to 10^5 M^{-1} (41, 42). Thus, the concentration of free ATP^{-4} is diminished by low concentrations of Mg^{++} ions. An experiment was performed in which total ATP and Mg^{++} were varied over a wide range such that the free ATP concentration was kept almost constant (Table VII) while $MgATP^{=}$ concentration varied widely. The free ATP concentration was calculated using a value of 40,000 M^{-1} for the binding constant[12]. The per cent inhibition, measured experimentally, is closely correlated to the free ATP concentration, while an increase in $MgATP^{=}$ from 0 to 0.8 mM has no effect on the degree of inhibition. The conclusion that $MgATP^{=}$ does not inhibit the enzyme is in agreement with the fact that other divalent anions at concentrations under 1 mM do not significantly affect the activity of the enzyme (Figure 4).

Changes in pH occurred upon the dilution of 100 mM ATP to 1 mM and upon mixing Mg^{++} and ATP, but the maximum change, up to 1 mM ATP, was found to be only $\pm$ 0.04 pH units, and is therefore negligible.

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[12] This value for the binding constant for Mg^{++} and ATP^{-4} was obtained by Burton (43) and is close to the values reported by other recent investigators (41, 44). Actually, the calculation of free ATP is little affected by fairly large changes in the binding constant.

TABLE VII

Effect of ATP and Mg^{++} on Yeast Fumarate Hydratase (F — M)

Total ATP (mM)	$MgATP^{=}$ (mM) calculated	Free ATP (mM) calculated	% inhibition
0.07	0	0.07	61
0.09	0.03	0.06	61
0.10	0.04	0.06	63
0.44	0.36	0.08	60
0.65	0.57	0.08	63
0.81	0.75	0.06	62
0.85	0.76	0.09	68
0.86	0.80	0.06	61

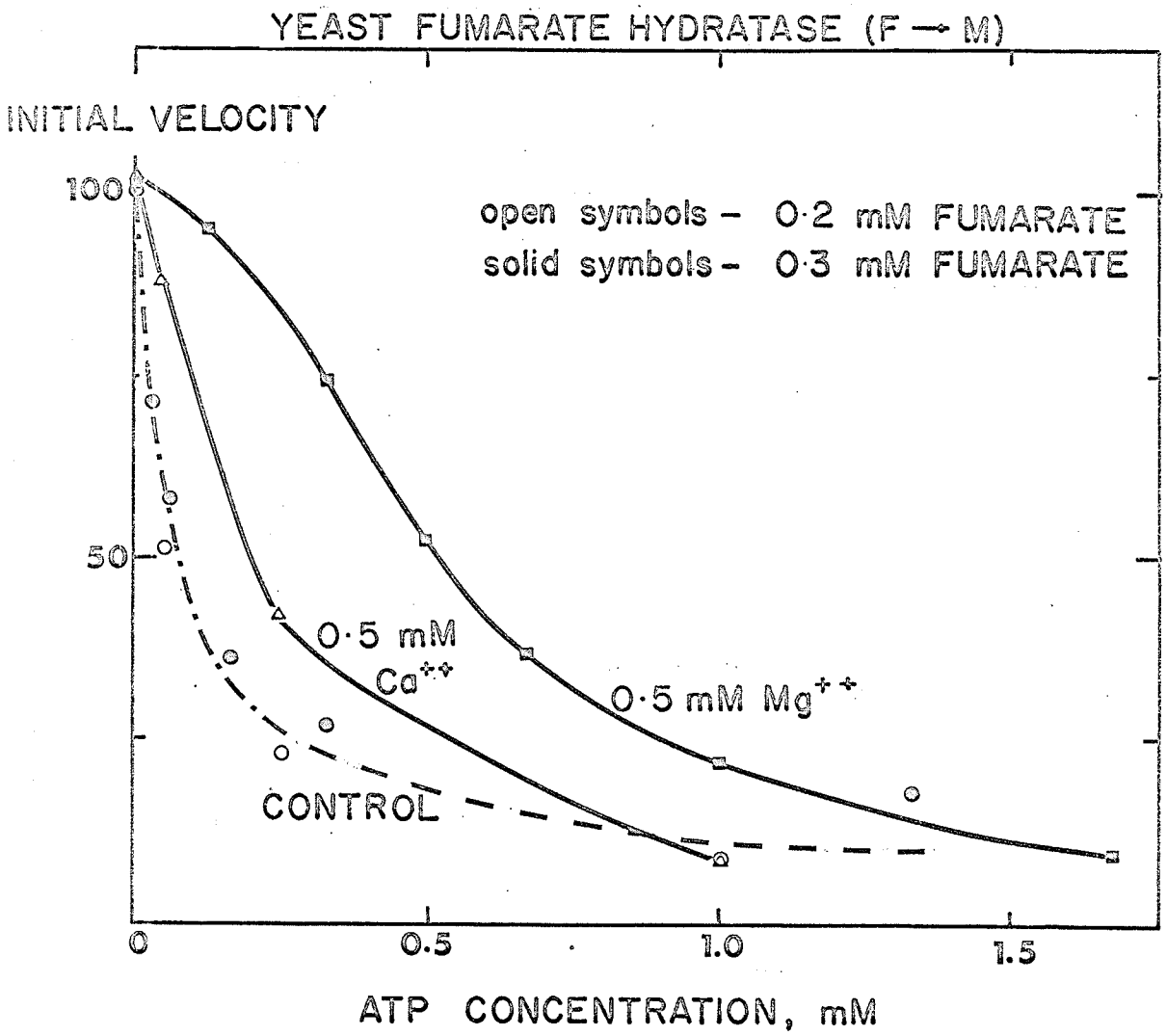

Figure 11. The effects of Mg^{++} and Ca^{++} on the inhibition of fumarate hydratase by ATP.

In these calculations no correction was made for the degree of ionization of ATP, although ATP is only 76% in the tetravalent form at pH 7, because the uncertainty in the assumed value of the binding constant is greater than 25%.

iii) ATP and GTP inhibition in the presence of a constant Mg^{++} or Ca^{++} level: On the basis of the conclusion that $MgATP^{=}$ does not inhibit the enzyme, increasing the ATP concentration in the presence of a constant amount of Mg^{++} (a condition which might exist in the cell) would be expected to result in a sigmoid shaped curve relating inhibition to total ATP concentration, a relationship which would have important regulatory implications. To test this prediction, the GTP concentration was varied from 0 to 0.5 mM in the presence of 0.5 mM Mg^{++} or Ca^{++} (Figure 9)[13]. In Figure 10 the effect of varying ATP concentration in the presence of 5 mM Mg^{++} is seen. Figure 11 is similar to Figure 9, only here the ATP concentration is varied in the presence of Mg^{++} and Ca^{++}. It is seen that Mg^{++} is about twice as effective as Ca^{++} in reversing the inhibition by ATP. This is in agreement with the fact that the binding constant for Mg^{++} and ATP^{-4} is approximately twice that for Ca^{++} and ATP^{-4}. These results support the conclusion that only free ATP inhibits and that $MgATP^{=}$ and $CaATP^{=}$ have little effect on the enzyme activity.

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[13] The higher control velocity in the presence of Mg^{++} and Ca^{++} is partly due to the pH effect described earlier (see section f(i)).

TABLE VIII

Effect of $Mg(acetate)_2$ and $Ca(acetate)_2$ on Inhibition of Fumarate Hydratase by Citrate (Yeast Enzyme, F—M)

Total citrate (mM)	% Inhibition			
	Control (no added salts)	$Mg(acetate)_2$ 9.1 mM	$Ca(acetate)_2$ 9.1 mM	Free citrate
0	0	0	0	0
3.6	73	31	43	0.2 mM
9.1	81	80	79	1.6 mM
27.3	88	92	91	19 mM

Fumarate concentration = 0.5 mM.

iv) Effect of Mg^{++} and Ca^{++} ions upon inhibition by citrate: Since Mg^{++} and Ca^{++} both bind citrate with an association constant of 3,000 M^{-1} $\pm$ 50% (43, 44), the effect of these ions upon the inhibition by citrate was examined. The binding constant of 3,000 M^{-1} is much lower than the MgATP binding constant (40,000 M^{-1}) and a correspondingly smaller effect upon the inhibition might be expected. The results in Table VIII show that Mg^{++} and Ca^{++} diminish the inhibition by citrate substantially. The data in Table VIII are not accurate because the Control (no added salts) had no added acetate ion, and therefore was only about one-half as active as the Mg and Ca controls. Since 9.1 mM $Mg(acetate)_2$ or $Ca(acetate)_2$ stimulate the enzyme, part of the effect of these salts could possibly be due to the acetate ion. Nevertheless, significant reversal of the citrate inhibition is obtained when the divalent cations are at a higher concentration than the citrate.

II. PIG HEART FUMARATE HYDRATASE: A COMPARISON WITH THE YEAST ENZYME

g) Source and Stability of the Enzyme:

Pig heart fumarate hydratase was a gift from Dr. R.A. Alberty. It was prepared by a salt fractionation procedure (9) and stored as the crystalline suspension in 50% $(NH_4)_2SO_4$ at 0° C. For these experiments a sample of the enzyme was washed with water (2 µl crystalline suspension in 500 µl deionized water) by centrifugation. The washed crystals were dissolved in 10 mM tris acetate + 15 mM EDTA (pH 7) at a

dilution of 1/1000 or greater. The diluted enzyme was stored frozen in glass tubes until used (-20° C).

The enzyme is unstable in 10 mM tris at 0° C (3), losing about 30% of its activity after 2 hours. In the present study, the addition of 15 mM EDTA to the enzyme improved the stability, so EDTA was used in all experiments that did not require Mg^{++} or Ca^{++} in the assay medium.

In order to correct for loss of activity during long experiments, a standard assay was run several times during the day, and velocities were corrected according to the loss of activity in the standard assay. When the loss in activity was very great, as occurred when very dilute enzyme solutions were used, only assays that were run in rapid sequence are compared; reproducibility is then better than $\pm$ 5%.

The assay procedure is the same as that described for yeast fumarate hydratase in sub-section (b).

h) Anion Effects on the Forward Reaction:

i) Chloride and acetate: Massey (2) has reported simple non-competitive inhibition of pig heart fumarate hydratase by chloride, bromide, iodide, and thiocyanate in 60 mM phosphate buffer at pH 6.35. Massey and Alberty (21) show (forward reaction) that changing the pH from below 7 to above 7 changes the inhibition by thiocyanate to activation; this activation occurs only at low thiocyanate concentrations while at high concentrations inhibition is observed. The effect of chloride and of acetate obtained in this study are

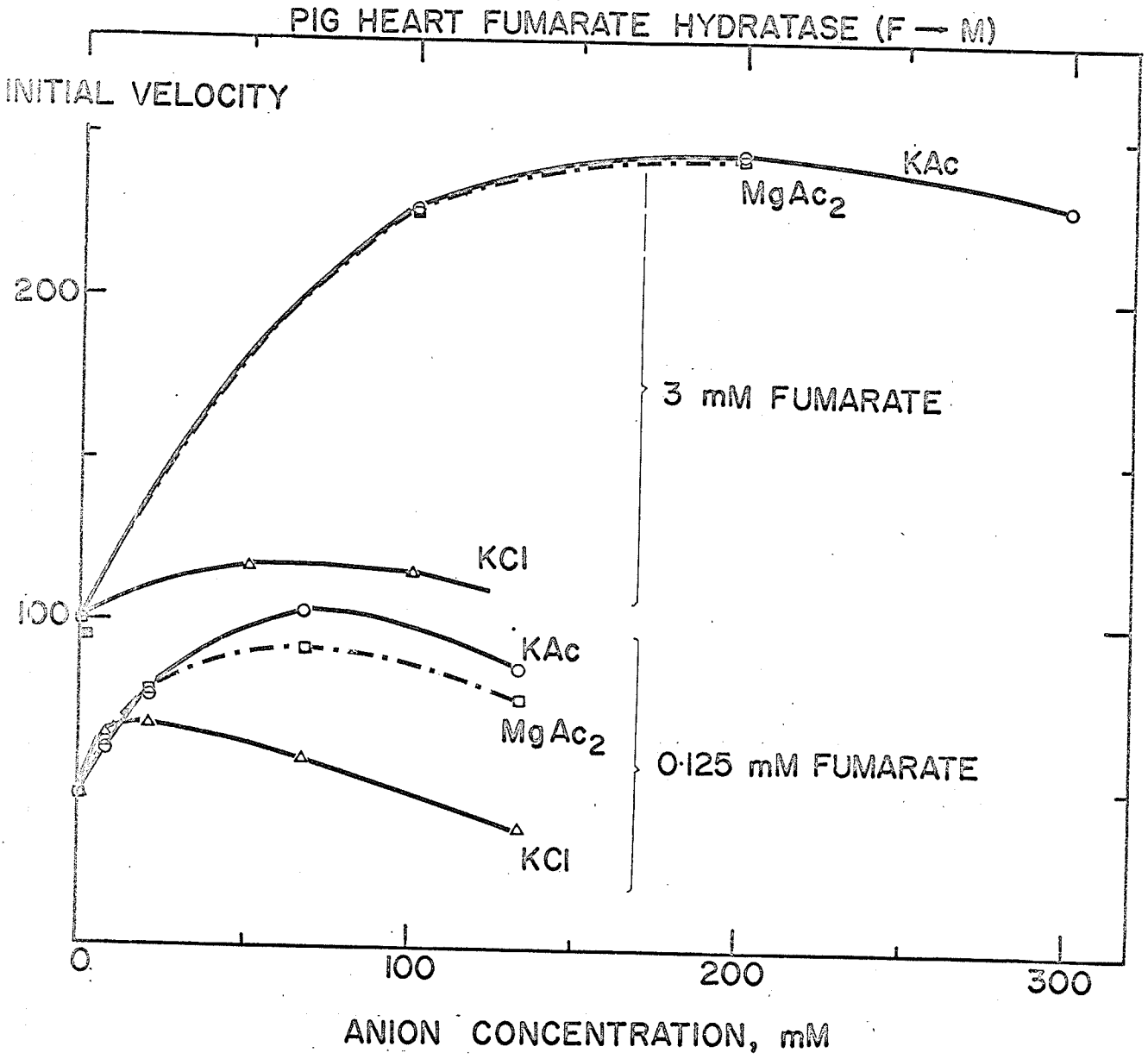

Figure 12. Activation of fumarate hydratase by chloride and acetate ions.

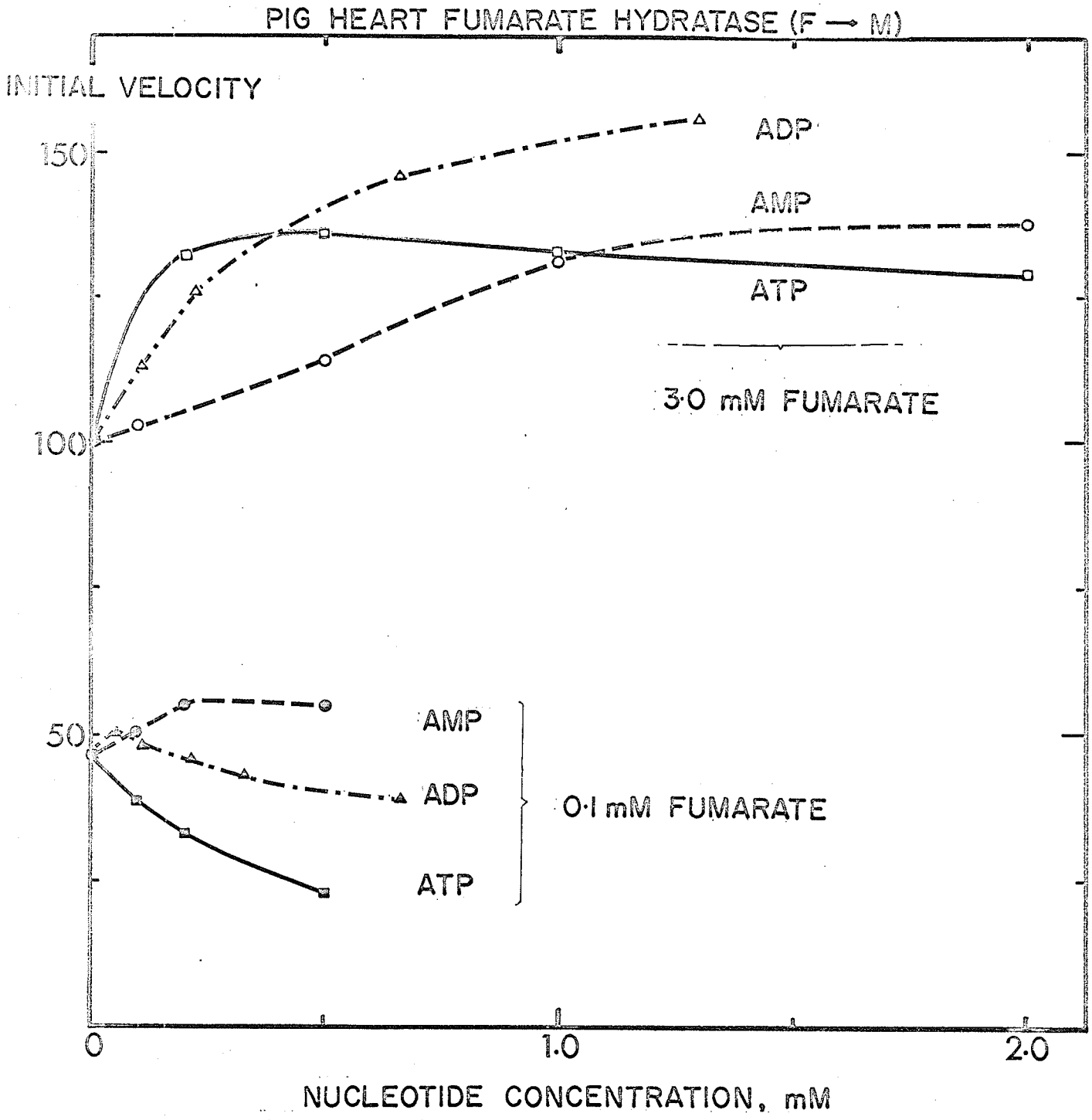

Figure 13. The effects of AMP, ADP, and ATP on the activity of fumarate hydratase.

seen in Figure 12. Chloride is a much less effective activator of the pig heart enzyme than of the yeast enzyme (cf. Figure 1). In contrast, acetate ion affects both enzymes to almost exactly the same extent (Figures 1 and 12). At very high concentrations both anions inhibit the enzyme, as in the case of the yeast enzyme. Na propionate and K isobutyrate were also tested and found to affect the pig heart enzyme in a manner very similar to the effect of acetate ion on that enzyme (forward reaction).

ii) Polyvalent anions: Inhibition by anions has been discussed in the Literature Review. Citrate is reported to be a competitive inhibitor of pig heart fumarate hydratase ($K_i = 3.5 \times 10^{-3}$ M in both forward and reverse directions) in 60 mM phosphate buffer (22), but an activator of the salt-free enzyme (M—F) (2). Succinate, D-, L-, and meso-tartrate are all competitive inhibitors (3) in 10 mM tris acetate. The similarity of the phosphate effects on the two enzyme preparations has been discussed in c(iv). Adenine nucleotides were found to affect the pig heart enzyme differently than they affected the yeast enzyme. In Figure 13 are shown the effects of AMP, ADP, ATP under the same conditions that were used for studying the yeast enzyme: pH 7.0 and $\mu = 0.01$ mM. At high fumarate concentrations AMP, ADP, and ATP all stimulate somewhat. At low fumarate concentrations, AMP stimulates, ADP stimulates at low concentrations

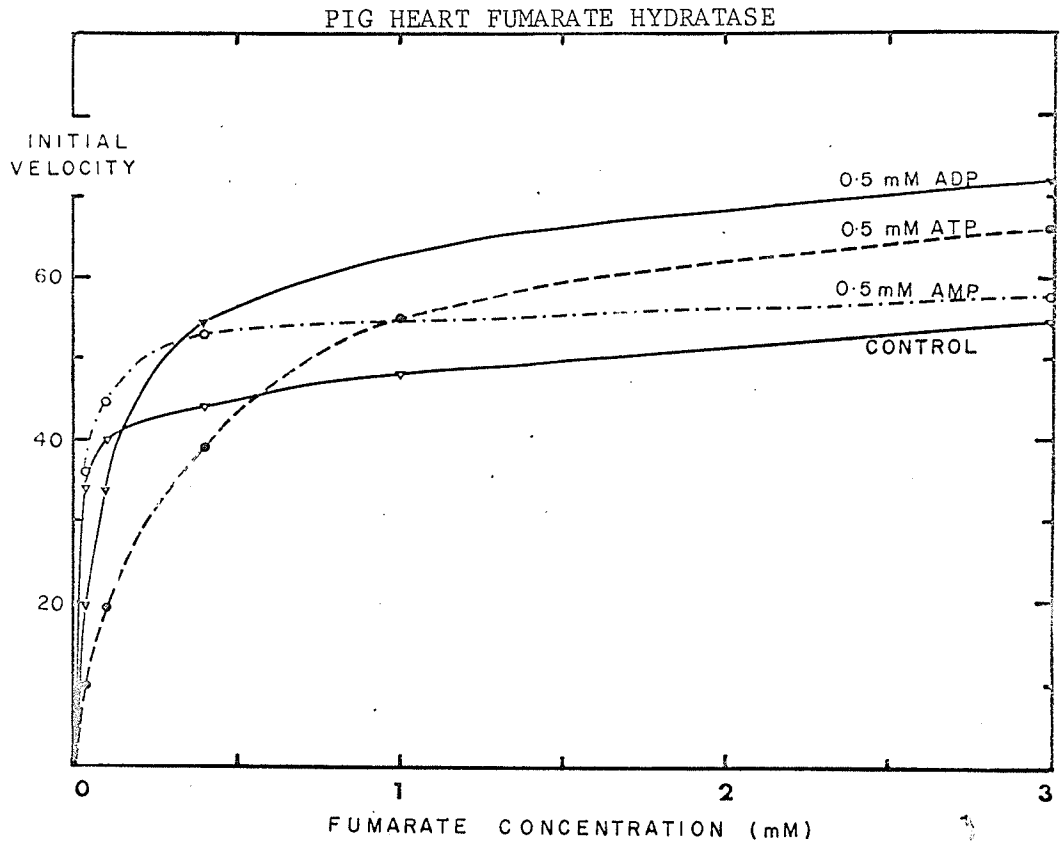

Figure 14. The effects of 0.5 mM AMP, ADP, and ATP on the activity of fumarate hydratase as a function of fumarate concentration.

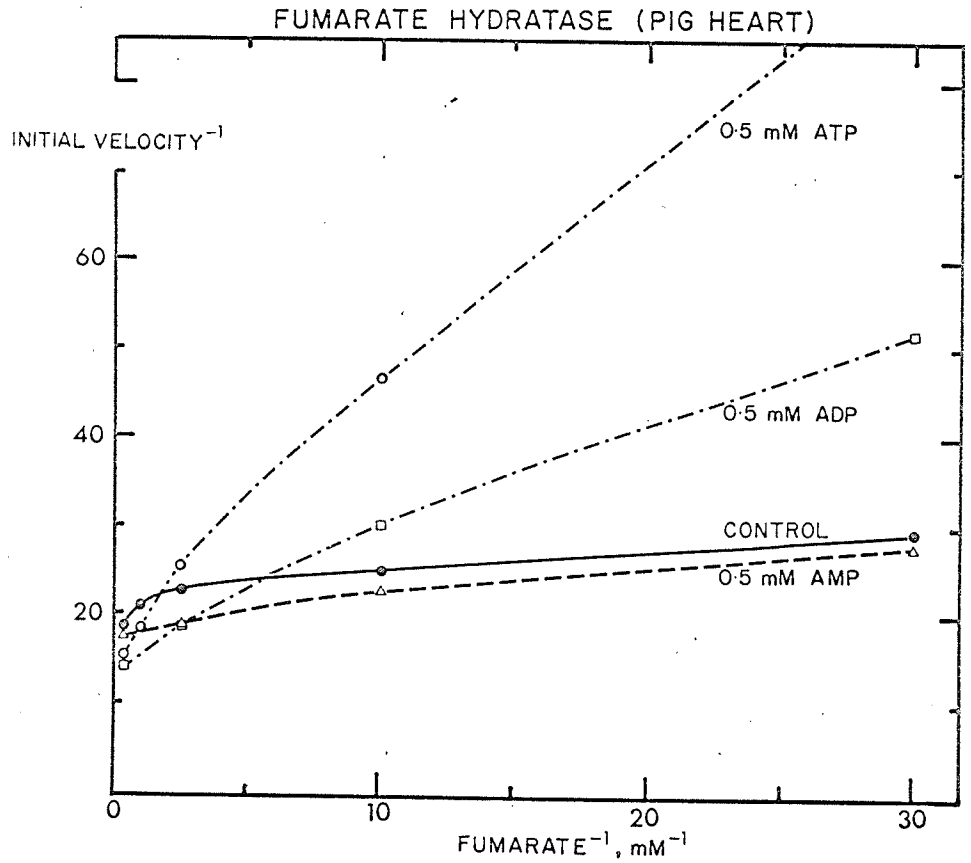

Figure 15. Lineweaver-Burk plot showing the inhibition of fumarate hydratase by ADP and ATP.

and inhibits at high concentrations, and ATP inhibits at all concentrations (compare to Figure 5).

iii) Dual effect of the nucleotides: As seen above, ADP and ATP may activate or inhibit, depending on the concentrations of fumarate and nucleotide. Figure 14 shows the effects of 0.5 mM AMP, ADP, and ATP over a 100-fold range of fumarate concentration. Under these conditions AMP only activates while both ADP and ATP inhibit at low fumarate concentrations and activate at high fumarate concentrations.

The reciprocal plots of the same data (Figure 15) illustrate the substrate activation of pig heart fumarate hydratase as already discussed in the Literature Review. From the linear portion of the control line (low fumarate) the K_m of the forward reaction is calculated to be 0.008 mM (computer fit to the 2/1 function). The adenine nucleotides at 0.5 mM concentration do not eliminate substrate activation, as seen by the curvature of the reciprocal plots in Figure 15.

The inhibition constants for ADP and ATP may be estimated from the slopes of the reciprocal plots (Figure 15). The K_i's (at 0.5 mM concentration of inhibitor) calculated by assuming simple competitive inhibition[14] are:

ADP - - - 0.14 mM

ATP - - - 0.051 mM

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[14] In simple competitive inhibition the slope of the Lineweaver-Burk plot is increased by the factor $(1 + I/K_i)$ and therefore the K_i is equal to the increase in slope divided by the concentration of inhibitor (see the calculation of K_i in j(ii)).

TABLE IX

Effect of P_i, AMP, ADP, and Mg^{++} on the Inhibition of Fumarate Hydratase by ATP (Pig Heart Enzyme, F — M)

Added compound (mM)	ATP conc. (mM)	Fumarate conc. (mM)	% Inhibition without added compound	% Inhibition with added compound
3 mM P_i	0.50	0.1	49	49
0.25 mM AMP	0.25	0.1	38	38
1.0 mM AMP	0.1	0.05	24	33
1.0 mM AMP	0.5	0.05	62	64
2.0 mM AMP	0.5	0.05	62	59
0.25 mM ADP	0.25	0.1	38	42
0.5 mM Mg^{++}	0.5	0.1	49	12
0.5 mM Mg^{++}	0.5	0.3	15	5% stimulation
0.5 mM Mg^{++}	3.0	0.3	45	46

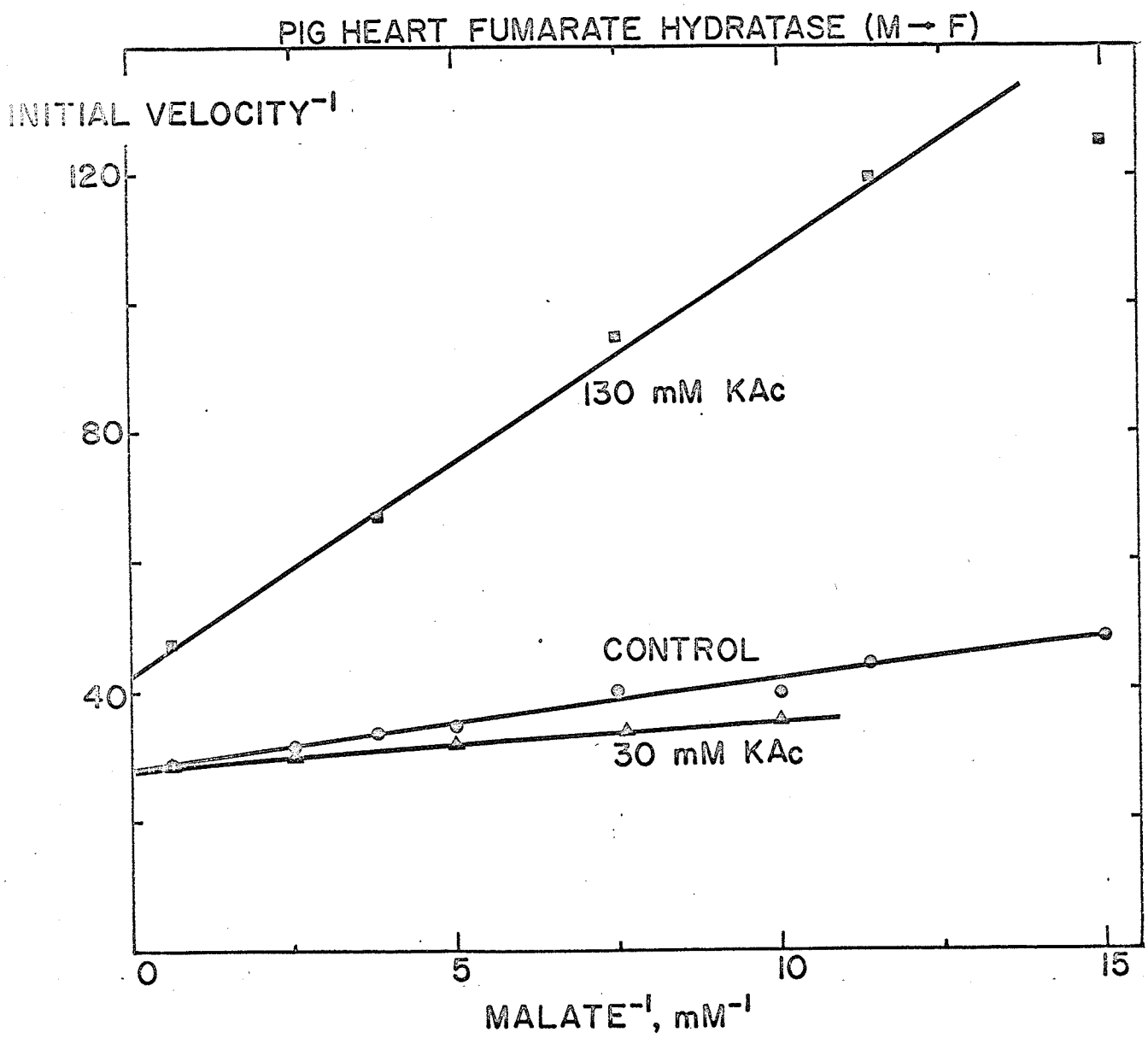

Figure 16. Lineweaver-Burk plot showing the slight stimulation by 30 mM K acetate and the inhibition by 130 mM K acetate.

Since the inhibition is not simple competitive, as seen from the curved replots of slope vs. ATP concentration (Figure 8), the calculated K_i depends on the concentration of inhibitor. The K_i at low ATP concentration is therefore less than the K_i calculated at 0.5 mM ATP. Evidence for this is seen in subsection j(ii) (reverse reaction) where the K_i calculated for 0.1 mM ATP is approximately one-half that of the K_i calculated for 0.4 mM ATP. If one assumes this to be true also for the forward reaction, the K_i at low ATP is approximately 0.025 mM.

iv) The effects of P_i, AMP, ADP, and Mg^{++} on the inhibition by ATP: Experiments were done to determine whether P_i, AMP, ADP, or Mg^{++} reversed the ATP inhibition of pig heart fumarate hydratase. The results are shown as per cent inhibition in Table IX because too little data was obtained to determine the ATP concentration required for 50% inhibition as was done for the yeast enzyme. No effect upon inhibition is observed with P_i, AMP, or ADP. The presence of Mg^{++} decreases the inhibition in a manner indicating that $MgATP^{=}$ does not inhibit the enzyme, but may actually have a stimulatory effect. AMP appears to enhance the inhibition by ATP at low ATP concentration, but has no effect at high ATP concentrations.

j) The Reverse Reaction (M → F):

i) Non-competitive inhibition by acetate: In Figure 16

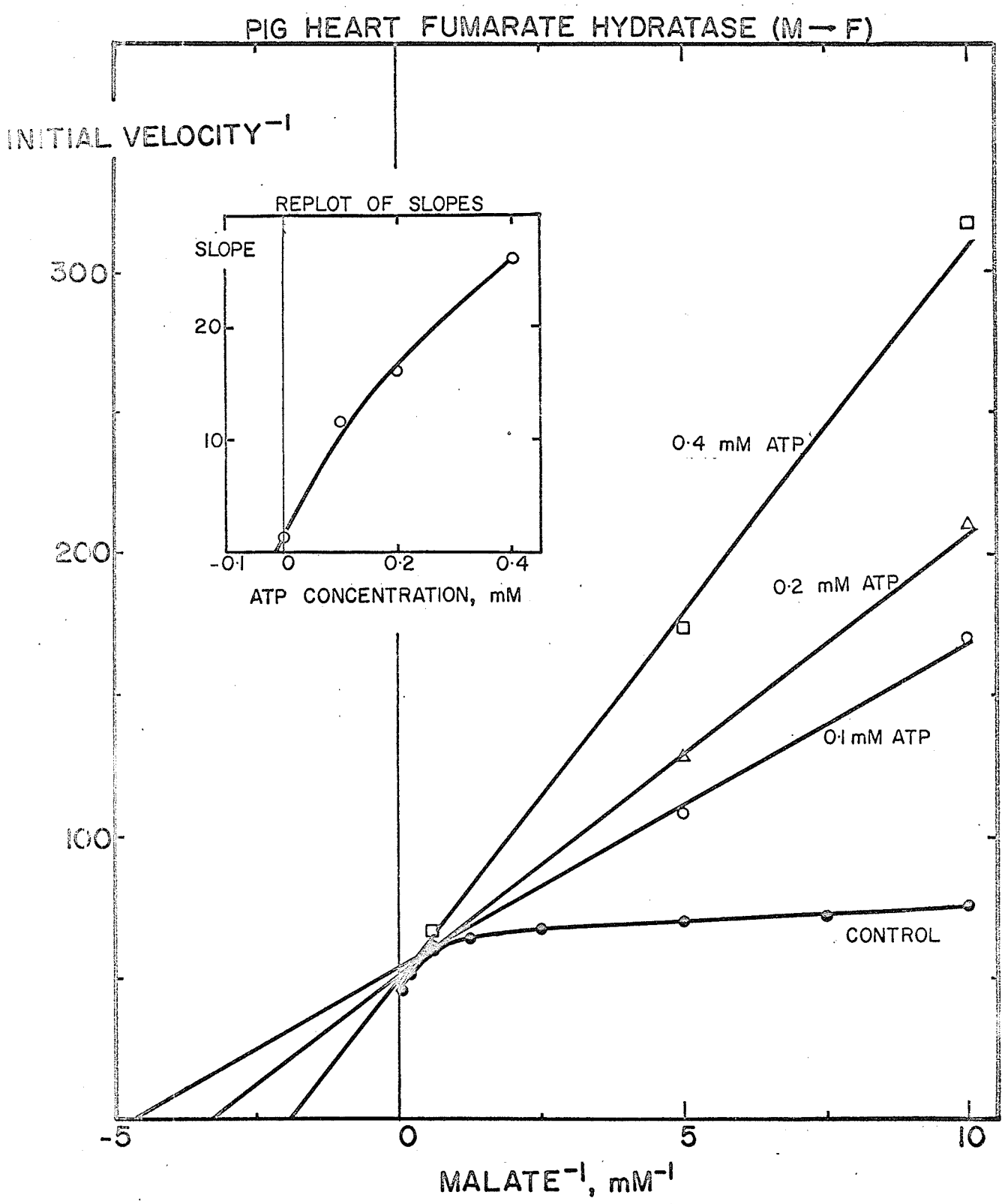

Figure 17. Inhibition of fumarate hydratase by ATP. The inset shows the replot of the slopes of the reciprocal plots.

the effect of acetate at two concentrations is seen. The reciprocal plots are drawn to illustrate the non-competitive inhibition at 130 mM K acetate and the slight activation by 30 mM K acetate. It is interesting that 130 mM K acetate is a potent inhibitor in this case and yet activates 90-140% in the forward direction (Figure 12).

ii) Inhibition by ATP: The effect of ATP on the rate of the reverse reaction is shown in Figure 17. ATP inhibits the reverse reaction with no sign of stimulation at any concentration of ATP or fumarate tested. Thus ATP, like acetate inhibits in a range of concentrations in which it stimulates the forward reaction. This inhibition, however, appears to be competitive with malate, whereas the acetate inhibition is non-competitive. The inset in Figure 17 shows the curved replot of slope vs. ATP concentration. The value of K_i obtained from the x-intercept of the replot is between 0.01 and 0.02 mM, but a great uncertainty exists, due to the low slope of the line for the uninhibited reaction, and the curve in the replot. The K_i may be calculated from Equation 15:

$$\text{when } I = 0,\ (\text{slope})_o = K_m/V_{max} \tag{15a}$$

$$\text{when } I = 0.1 \text{ mM ATP},\ \text{slope} = (K_m/V_{max})(1 + 0.1/K_i) \tag{15b}$$

The increase in the slope of the reciprocal plot, due to the presence of 0.1 mM ATP is seen to be equal to $0.1/K_i$, or I/K_i in the general case. The K_i's calculated from the slopes

(Figure 17) are:

0.1 mM ATP, K_i = 0.0105 mM

0.2 mM ATP, K_i = 0.015 mM

0.4 mM ATP, K_i = 0.018 mM

The increase in the calculated K_i as the ATP concentration is increased from 0.1 mM to 0.4 mM supports the assumption of a two-fold change in K_i between 0 and 0.5 mM ATP (see section h(iii) above) for the forward reaction. The K_i values determined from the data in Figure 16 are subject to the same error as the K_m calculated from these data (see below).

The K_m for the reverse reaction is 0.035 mM calculated from the data in Figure 16, and 0.016 mM from the data in Figure 17. Both values agree with Alberty's value of 0.017 mM (11) within experimental error. The accuracy of this determination is poor because the lowest malate concentrations which were used are greater than 4 K_m's[15].

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15 An accurate determination of the K_m can only be obtained when working with substrate concentrations in the vicinity of the K_m (approximately 0.2 to 5 K_m's).

D. DISCUSSION

The results of this study have a bearing upon the mechanism of anion effects and possible regulatory importance of anion effects upon the activity of fumarate hydratase. These two topics will be discussed in that order in this section of the thesis.

a) Anion Effects and Possible Mechanisms of Action:

i) Effects of anions: Several generalizations may be drawn concerning the effects of anions on fumarate hydratase activity. These conclusions are based on observations made with both the yeast and the pig heart enzymes and must be compatible with any proposed mechanism of action of anions on the enzyme. The general conclusions are given below and include a discussion of specific examples.

1) Every anion produces a specific effect on the enzymic activity although closely related anions may be quite similar in their effects under certain experimental conditions. This specificity is substantiated by the effects of numerous other anions studied by other authors and suggests that specific enzyme sites are involved as has been suggested by both Alberty (1) and Massey (2).

2) Most anions can both stimulate and inhibit fumarate hydratase activity, depending on the substrate concentration and on the anion concentration. With pig heart enzyme, all anions tested stimulate at low concentrations and inhibit at high concentrations. As a result, at each substrate concentration there is an optimal anion concentration, the magnitude of which differs for different anions. This optimal anion concentration is increased

as the substrate concentration is increased. It appears that the maximal stimulation which an anion may produce depends on its potency as an inhibitor as well as its effectiveness as an activator and that high substrate concentrations, by suppressing the inhibition competitively, enhance the manifestation of the stimulatory effect. In the case of the yeast enzyme stimulation and inhibition were obtained only with anions of charge -2 or less; with anions having a charge greater than -2 only inhibition has been observed even at very low concentrations.

Activation and inhibition effects by a single anion must be due either to the binding of the anion at two separate sites or to two forms of the enzyme. The overall effect, then, would seem to depend on the relative affinities for the two sites (or two enzyme forms) (discussed in #6).

3) Both substrates (fumarate and malate) appear to activate at intermediate and inhibit at very high concentrations. The simplest assumption, which has been made by other investigators, is that substrate activation is a special case of activation by anions (see Literature Review) and therefore it is not treated separately from other anion effects in the mechanisms proposed below. The strongest evidence for this conclusion is that the V_{max} of the substrate activated enzyme is the same as the V_{max} in the presence of 50 mM phosphate or 100 mM KCl (yeast enzyme, F → M).

The study of other anion effects is hampered by substrate activation. It would seem that low substrate concentrations (in the range where substrate activation does not occur) would be best suited for the study of stimulation effects. However, at low substrate concentrations inhibition by anions is much more marked than at high substrate concentrations due to the competitive nature of the inhibition. The use of a wide range of substrate concentrations was thus essential for an understanding of the activation effects. It was found that where an anion exhibited a dual effect, inhibition was most pronounced at the lowest substrate concentrations used (for most experiments 0.1 mM to 0.5 mM) and stimulation was most pronounced at high substrate concentrations (1 mM to 10 mM). The substrate inhibition at very high concentrations of fumarate or L-malate was not studied.

4) The optimal anion concentration for stimulation depends in certain cases upon the direction of the enzymic reaction. With the pig heart enzyme, acetate stimulates the forward reaction (F→ M) at concentrations up to 60 mM for 0.1 mM fumarate and 200 mM for 3.0 mM fumarate, but inhibits the reverse reaction markedly at 130 mM over a very wide range of fumarate concentrations. Similarly, ATP inhibits the enzyme in the forward direction at low fumarate concentrations and stimulates at high fumarate concentrations, but inhibits the enzyme in the reverse direction at all fumarate and ATP concentrations. This result is similar to that reported for phosphate by Alberty et al (1).

5) Anion effects appear to be related to the charge and structure of the anion. Anions with a charge of -1 activate over a wide range of concentrations (Figure 4). Anions that are more negative, such as sulfate and phosphate, are more potent both as stimulators and as inhibitors of fumarate hydratase. However, the inhibitory effect is enhanced more than the stimulatory effect, so that the maximum stimulation obtained with these anions is lower than that obtained with uni-valent anions and occurs at lower concentrations. The similarity between the effects of anions with similar ionic charge is quite pronounced, e.g. chloride and acetate, or sulfate and phosphate (Figure 4). As mentioned above, anions with the greatest negative charge are the most potent inhibitors. However, it appears that the number of negative charges of the anion is not the sole factor in determining the affinity (or potency) of inhibitors. Thus, of three anions that are triply charged at pH 7, citrate and PP_i are much more potent inhibitors of the yeast enzyme than is EDTA. Similarly, the nucleoside triphosphates are almost 10 times more potent as inhibitors than is adenylosuccinate, despite the fact that both have a charge of -4. It may be concluded from these studies that the density of negative charge is important. The size of the compound is of little importance as seen in the similar potencies of PP_i and ADP, both triply charged anions with a pyrophosphate group, but differing greatly in size. The finding that polyvalent anions and especially the polyphosphates are potent

TABLE X

The Effect of Phosphate on Yeast Fumarate Hydratase in the Absence and Presence of 50 mM KCl

Phosphate concentration	Relative Activity	
	No KCl	50 mM KCl
0	10.5	30.5
1 mM	11.5	27.5
3 mM	14.0	22.5
10 mM	9.0	11.5

Fumarate concentration = 0.1 mM.

inhibitors of the enzyme suggest that the inhibiting anion binds by electrostatic attraction to several closely clustered positive groups on the enzyme surface.

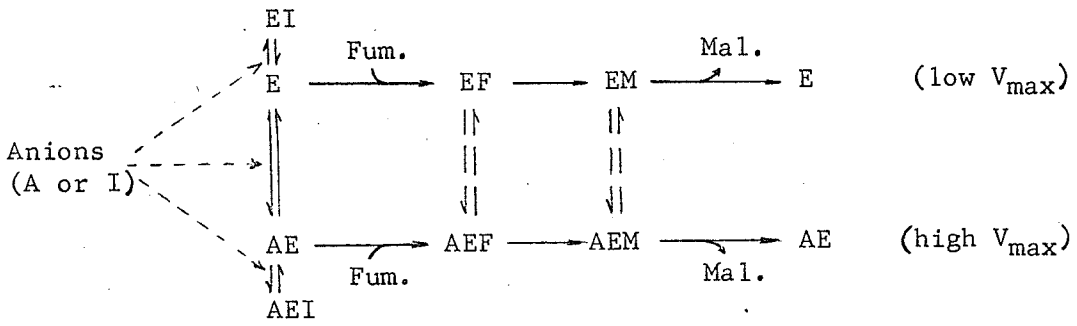
Competitive inhibition by substrate analogues was not investigated in the present study and thus will not be discussed here. Inhibition by these compounds is closely related to their structure (3), suggesting that these inhibitors act by binding at the substrate-binding site.

(6) When an activator is present in saturating concentrations it prevents further stimulation by another anion. As described in #3 above, at high substrate concentrations the substrate appears to saturate the stimulatory sites and thus prevent further stimulation from occuring. Other activating anions have a similar effect. Thus, when phosphate was tested in the presence of 50 mM KCL, it acted only as an inhibitor and the stimulatory effect at low concentrations was not observed (Table X). Similarly citrate has been found to activate the salt-free enzyme (2) but to inhibit the enzyme in the presence of 60 mM phosphate buffer (22).

It is difficult to obtain a true estimate of the effectiveness of an anion as an activator because all of the anions tested also showed net inhibition at sufficiently high concentrations. Thus, although all triply charged anions only inhibited the yeast enzyme (F → M), they might in fact also bind at the activating site with similar affinities as do monovalent anions.

7) Both competitive and non-competitive inhibition effects are observed. The majority of anions tested showed competitive inhibition (eg. ATP, P_i, ADP). Other anions also have been reported (22) to produce competitive inhibition. However, 130 mM acetate is a non-competitive inhibitor with respect to malate, and similar results were found with chloride, bromide, iodide, and thiocyanate (see Literature Review). These two types of inhibition are further discussed below with reference to the possible mechanisms of the ionic effects.

ii) Possible mechanisms of the ionic effects: the observations discussed above are not sufficient to establish the mechanism with certainty. Although any mechanism which is postulated must be compatible with all of the known observations, this still leaves much room for speculation. Two mechanisms are discussed below. The first model is a modification of Alberty's (1) mechanism for the phosphate effect:



(16)

where A is an anion acting as an activator, I is the same anion acting as an inhibitor, F is fumarate and M is L-malate. This mechanism is similar to Mechanism 4 (described in the Literature

Review) if one substitutes A or I for the S and the B of Mechanism 4. No assumption is made about the number of molecules of anion bound. That is, the number of activation or inhibitory sites is represented as a single site for convenience. The mechanism as written shows the effect of one anion on the enzyme, i.e. the general case where activation and inhibition both occur. This anion may be the substrate or one of the added test compounds. The mechanism is consistent with the dual effect exhibited by one anion (described in the Literature Review), and the same explanation is applied to all other anions having a similar effect. This treatment includes the two special cases where only activation or only inhibition are produced by an anion. This mechanism is compatible with anion effects such as substrate activation, activation by other anions, and competitive inhibition by anions but does not explain the fact that acetate, at concentrations which stimulate the forward reaction, inhibits the reverse reaction or the fact that this inhibition is non-competitive. In order to explain non-competitive inhibition one must assume that the inhibition by some anions occurs not only by combination with free enzyme in a manner competitive with malate, (shown by writing I on the right of E), but that an additional inhibitor binding site exists which is not shown in Mechanism 16. Non-competitive inhibition must necessarily involve the combination of inhibitor with an enzyme form other than the form which binds substrate. Thus, reactions of the type:

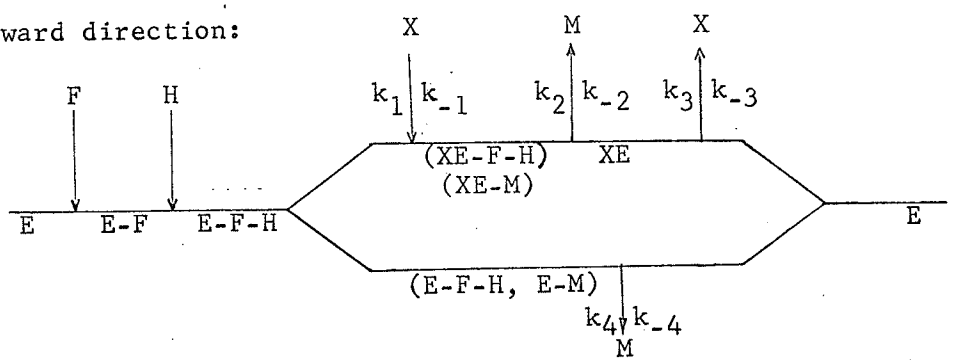
$$EF + I \rightleftharpoons EF\text{-}I \quad \underline{or} \quad AEF + I \rightleftharpoons AEF\text{-}I \tag{17}$$

must be included in the reaction mechanism for these special cases.

According to Mechanism 16, the effects of anions may be classified as those which only inhibit the enzyme by acting only as I and those which act as A or I to activate or inhibit. For example the effects of nucleoside polyphosphates would be explained according to this mechanism by assuming that the nucleosides can act as A or I on the pig heart enzyme but only as I on the yeast enzyme. The equilibria shown by the dashed arrows in Mechanism 16 are not necessary to the explanation of the ionic effects. However, they open up the possibility of alternate pathway mechanisms occuring.

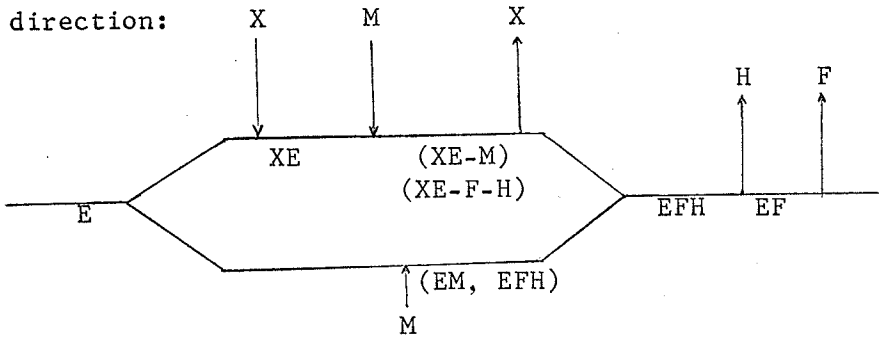
The second model to be discussed can be derived from the above mechanism by setting certain rate constants to zero. This mechanism is:

Forward direction:



(18)

Reverse direction:



(18a)

where H is water, F is fumarate, M is L-malate, and X is the anionic effector. It is assumed that all of the rate constants of the upper

path are faster than the slowest rate constant of the lower path, i.e. the presence of X on the enzyme speeds up the rate-limiting step.

This mechanism is attractive because it is simple and yet is compatible with many of the observed effects of anions on the fumarate hydratase reaction. An anion, X, would stimulate by shifting the reaction to the upper, faster path. However, it would also be a product inhibitor by backing up the last reaction of the upper path. In the forward direction the inhibition would be competitive with fumarate since it results from a decrease in free enzyme E, the form with which fumarate combines. This would account for the facts that low concentrations of anions activate the enzyme and high concentrations inhibit, the optimal anion concentration and maximal activation increasing with increasing fumarate concentration. At sufficiently high concentrations, however, fumarate would act as X and so would activate the enzyme itself. Similar considerations apply to the reverse reaction, except that the inhibition would result from combination of X with EFH and thus would not be competitive with respect to malate (unless the release of H and F were so rapid as to keep EFH in equilibrium with E). Activation in this direction, on the other hand, would be competitive with respect to malate.

The specific effects of a given anion would depend on the affinity of the anion for the enzyme, and also on the relative affinities for the two enzyme forms which it binds, namely EFH and E. If an anion binds to both forms with similar affinities, then

its effects on the initial rates of the two opposing reactions catalyzed by the enzyme will be similar: stimulation at low anion concentration and inhibition at high anion concentration. However, the maximal activation and the optimal anion concentration will be higher for the forward reaction (F → M, initial rate) since the anion is competitive in its inhibitory action in that direction, while it is competitive in its stimulatory action in the reverse direction. If, on the other hand, the anion were to bind one form of the enzyme more strongly than the other, it would be predicted that the maximum stimulation in the forward and reverse directions would be different. For example, if X had a higher affinity for E-F-H than for E, the forward reaction would be stimulated over a wider range of anion concentrations than the reverse reaction (as found for the effect of acetate on the pig heart enzyme). Were the affinity for E-F-H very much higher than for E, only inhibition of the reverse reaction might be expected, as found for the effect of ATP on the pig heart enzyme.

It must follow, from thermodynamic considerations, that if X were to have a greater affinity for EHF than for E in Mechanism 18, there would result an increase in the dissociation constant for M when the upper branch of the mechanism is followed[17]. This would

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17 The overall equilibrium is independent of the pathway followed by the reaction. Thus, $k_{-1}k_{-2}k_{-3}/k_1k_2k_3 = k_{-4}/k_4$ or, in terms of dissociation constants, $K_{EM} = K_{EX\text{-}M}(K_{EHF\text{-}X}/K_{E\text{-}X})$. If X has a higher affinity for E-H-F than for E, then the dissociation constant for M in the presence of X ($K_{EX\text{-}M}$) is greater than the dissociation constant in the absence of X (K_{EM}).

increase the K_m. Another effect on the apparent K_m would be produced as a result of the decrease in the free enzyme concentration, due to the formation of EX. No attempt is made to predict which of these two effects on the apparent K_m is more important. Thus, an anion may increase the V_{max} as described above, but in addition it may increase the K_m for one or both substrates to such an extent that no increase in rate is observed at certain substrate concentrations. This is most likely to occur at low substrate concentrations where the effect of increasing the apparent K_m would be most noticeable. This is consistent with the effect of ATP on the pig heart enzyme, where stimulation was observed at high substrate concentrations, but inhibition at low substrate concentrations for the forward reaction. It should be emphasized that other mechanisms similar to that represented in equation (18) might also be consistent with the experimental results. For example, the sequence of addition of fumarate and water indicated in the model is only one of the sequences compatible with the competitive relation between anion inhibition and fumarate. The addition could as well be random.

The main distinctions between the two models discussed above are:

1) The first model postulates the existence of separate stimulatory and inhibitory sites on the enzyme. In the second model inhibition occurs simply because not all enzyme forms of the reaction sequence can combine with the activating anion so that its release is an obligatory step of the sequence.

2) The second model accounts in a simple way for the non-competitive inhibition and competitive stimulation by acetate which was observed with the pig-heart enzyme in the reverse (M → F) direction. However, not all anions are non-competitive inhibitors with respect to malate. Phosphate (1) and (as shown in the present study) ATP, are competitive inhibitors of pig-heart fumarate hydratase in either direction.

It might well be that a single mechanism does not apply to inhibition by all anions. For example, the second model might account for activation by all activating ions and for inhibition by monovalent ions such as acetate, but there might also be a separate inhibitory site on the free enzyme for nucleotide triphosphates, to which inorganic phosphate can also bind with a low affinity.

It is tempting to suggest that an allosteric transition is responsible for the changes in kinetic properties of fumarate hydratase upon binding of an effector. The subunit structure of fumarate hydratase is compatible with the allosteric protein model discussed earlier in the thesis. The kinetics, however, do not fit the typical sigmoid shaped plot for velocity vs. substrate concentration which one would expect to see in the presence of inhibitor as described by Monod, Wyman, and Changeux (32). The present enzyme could well be active in both configurations, and thus not fit any of the situations that these authors treated. If the binding of an inhibitor or an activator results in a change in enzyme configuration, as the allosteric model predicts, it should be possible to detect this change experimentally. A possible experiment would be to determine the stability of the tetrameric

enzyme molecule to dissociation into subunits in the presence and absence of an inhibitor as was done by Changeux for L-threonine deaminase (45).

b) The Reversal of Inhibition by Divalent Cations: a Possible Regulatory Role of the MgATP Effect:

The most important observation, with regards to regulation, is that the activity of the enzyme depends on the concentration of free ATP which is only a small fraction of the total ATP (when Mg^{++} and ATP are at approximately equal concentrations as in Table VII). The sigmoid shaped relationship between enzyme activity and total ATP concentration may be of regulatory importance. Such a sigmoid curve suggests that it is the concentration of free ATP, over and above the ATP which is bound to divalent cations in the cell, that is responsible for the regulation of fumarate hydratase activity. Furthermore, the $MgATP^{=}$, which constitutes the major portion of the total ATP, is believed to be available as substrate for energy requiring reactions.

This theory is compatible with the observation that few, if any, enzymes use free ATP as a substrate. Most of the enzymes that have ATP as one of the substrates, also have an absolute requirement for a divalent metal cofactor (frequently this is Mg^{++}). The intracellular magnesium concentration is approximately 15 mM (mammalian muscle) and this is in the same range as the concentration of ATP in rat heart muscle (5 - 10 mM, ref. 45). The obvious advantage of having a regulatory system capable of responding to

small increases in the concentration of total ATP (i.e. when Mg^{++} and ATP concentrations are approximately equal, Figures 14 & 15) is that the enzyme activity will be inhibited quite sharply after a certain level of ATP (determined by the Mg^{++} concentration) has accumulated. If the ATP concentration now drops suddenly, the enzyme is returned to almost full activity while a relatively large fraction of the total ATP is still available as an energy reserve.

It has been mentioned earlier that several of the enzymes in the metabolic paths by which ATP is generated are inhibited by ATP, as was found for fumarate hydratase in this investigation. The observations reported in the literature for such enzymes (hexokinase, ref. 46, and phosphofructokinase, ref. 33) suggest that free ATP is the inhibitor rather than a metal-ATP complex, and this may also be true for other enzymes in these pathways. It is known that the substrate for several of the enzymes that use ATP is not free ATP, but the MgATP complex. That is to say, divalent cations can change the inhibitor of a pathway (ATP) into a substrate (MgATP). The possible regulatory role of the MgATP effect may be important in certain physiological phenomena which are described below.

1) Mitochondrial respiration is associated with the movement of divalent metal ions into the mitochondria (29). In vitro studies have revealed that certain salts are accumulated by mitochondria, and that this process may be accompanied by such affects as hydrogen

ion release, mitochondrial swelling, and stimulated phosphate-dependent respiration. With magnesium-phosphate accumulation there is a clear relationship between ion uptake and respiration (29, 40). The same is not true for potassium uptake. At low levels of K^+ there is no stimulation of respiration and at high levels the stimulation is small and not critically related to the K^+ concentration (33). The uptake of calcium or of magnesium ions by the mitochondria *in vitro* is associated with a release of hydrogen ions (29) in the presence of phosphate. When sulfate replaced phosphate no H^+ was evolved even though magnesium sulfate accumulation is known to occur under these circumstances (33). These results suggest that the movement of Mg^{++} into the mitochondria during respiration might be a factor in the inverse relationship known to exist between respiration and glycolysis, that is, the Pasteur and Crabtree effects.

2) Muscular contraction is believed to be triggered by the movement of calcium ions into the cytoplasmic sap. The endoplasmic reticulum of striated muscle is capable of binding considerable quantities of calcium ion (approximately 0.2 μmoles Ca^{++}/gm muscle in 30 msec., ref. 30). During relaxation of the muscle, the removal of calcium from the sarcoplasm is involved, just as the release of Ca^{++} is involved in the contraction process. An increase in glycolysis also occurs concommitant with muscular contraction (or stimulation), which endures only as long as muscle contraction lasts. This local increase in metabolism therefore

seems to be related to the movement of calcium ions, just as the contraction and relaxation of the muscle fibre are.

It is possible that the effects described above might be due to the local decrease in concentration of free ATP as divalent cations move from one compartment to another. The effect of divalent cations is possibly only one of several factors contributing to the regulation of glycolysis and the citric acid cycle. Two or more controls on the same system are desirable since better control and faster response is possible when both an increase in product (AMP) and a decrease in ATP are being watched simultaneously by the energy-producing systems of the cell.

TABLE XI

Molar Extinction Coefficients for Disodium Fumarate at 25°C in Dilute Phosphate Buffer

Wavelength	Molar extinction coefficient ($mM^{-1}cm^{-1}$)
205	16.6
210	15.1
215	12.45
220	9.30
225	6.50
230	4.52
235	3.30
240	2.44
245	1.88
250	1.45
255	1.15
260	0.90
265	0.705
270	0.535
275	0.393
280	0.278
285	0.184
290	0.118
295	0.074
300	0.0433
305	0.0247

These data are taken from reference 1.

E. APPENDICES

I. Experimental Procedures:

1) Assay procedure: The procedure for the assay of fumarate hydratase activity is described on page 25. The Cary Model 15 recording spectrophotometer was used to record the changes in optical density after the addition of the enzyme to the medium. It was possible to run two assays simultaneously and to record the two reaction rates by alternating two cuvettes in the light path for brief periods. The slope of the line (change in optical density/unit time), from which initial rates were determined, was obtained by drawing the best possible straight line through the tracing on the recorder chart.

In order to obtain initial rates within 5% of the true initial rate, the extent of the reaction was limited to no more than 7% of the forward reaction or 2% of the reverse reaction (see ref. 1). Spectral data for Na fumarate, obtained by Alberty (1), are given in Table XI. The sensitivity of the assay was varied by changing the wavelength in such a manner that the measured optical density change was approximately 0.1. Certain assays had to be done at a wavelength where the sensitivity was poor, e.g. when low substrate concentrations were used in the presence of nucleotides. In these cases a more sensitive slide-wire was used, on which the 0 - 0.1 portion of the direct scale was expanded to full scale.

2) ADP purification: The ADP used in the experiments with the yeast enzyme was obtained from P-L Biochemicals, Inc. and was found to contain 3% ATP. ADP from Sigma Chemical Co. was found to contain 1% ATP and approximately 3% AMP. Purification of the

Sigma ATP was attempted on a 1 cm x 4 cm column of Dowex 50W-X8 cation exchange resin. Approximately 80 μmoles ADP was put on the column in 1 ml of 0.05 N HCl. The ADP was eluted with deionized water at a flow rate of 1 ml/min. ADP (+ ATP) came off the column first and was neutralized to pH 7 immediately. No separation between ADP and ATP was obtained. The purified ADP contained 1% ATP and 0.5 % AMP. ATP was assayed as described in footnote 9, page 34. AMP was determined by chromatography on a 1 cm x 4 cm column of Dowex 1-X8.

II. Rate Equations:

1) Random bi-uni (Mechanism 7): The steady-state rate equation, when written in the reciprocal form, is a 2/1 function (ref. 15, page 95), i.e. (1/(S)) appears to the second power in the numerator and to the first power in the denominator. Cleland (23) has derived a rate equation for the rapid equilibrium bireactant mechanism in which it is assumed that all steps in the reaction sequence are very rapid except for the interconversion of the central complexes ((EFH) and (EM) in Mechanism 7):

$$v = \frac{V_1V_2(FH - M/K_{eq})}{K_{iF}K_HV_2 + K_HV_2F + K_FV_2H + V_2FH + V_1M/K_{eq}} \qquad (19)$$

where v = initial velocity; V_1 = maximum initial velocity in the forward direction (F → M); V_2 = maximum initial velocity in the reverse direction (M → F); F, H, M = concentrations of fumarate, water and malate respectively; K_F = Michaelis constant for fumarate; K_H = Michaelis constant for water; K_{iF} = inhibition constant for fumarate.

Haldane relationships for the rapid equilibrium bi-uni mechanism:

$$K_{eq} = \frac{V_1 \ K_M}{V_2 \ K_F \ K_{iH}} = \frac{V_1 \ K_M}{V_2 \ K_{iF} \ K_H} \qquad (20)$$

where K_M = Michaelis constant for malate; K_{iH} = inhibition constant for water; K_{iF} = inhibition constant for fumarate; all other constants are as defined above.

2) Ordered bi-uni (Mechanism 8): The steady-state rate equation derived by Cleland's method (23) is:

$$v = \frac{V_1V_2 \ (FH - M/K_{eq})}{K_{iF}K_HV_2 + K_HV_2F + K_FV_2H + V_2FH + V_1M/K_{eq} + V_1HM/K_{iH}K_{eq}} \qquad (21)$$

where the symbols are as defined for Equations 19 and 20.

Haldane relationships for the ordered bi-uni mechanism:

$$K_{eq} = \frac{V_1 \ K_M}{V_2 \ K_{iF} \ K_H} = \frac{V_1 \ K_{iM}}{V_2 \ K_F \ K_{iH}} \qquad (22)$$

where the constants are as defined above.

The equations for Mechanism 9, where water adds to the enzyme before fumarate, have the same form as Equations 21 and 22 with F and H, K_F and K_H, and K_{iF} and K_{iH} interchanged.

3) Uni-uni mechanism: The rate equation and Haldane relationship for a uni-uni mechanism have been given earlier on page 16 (Equations 11 and 12).

4) Partial allosterism (see page 58): It is assumed that the substrate (S) is an activator and both forms of the enzyme, E and ES, have activities:

$$v = \frac{V_1K_1Ka_2S + (V_1K_1 + V_2Ka_1)S^2 + V_2S^3}{K_1Ka_1Ka_2 + (K_1Ka_1 + K_1Ka_2 + Ka_1Ka_2)S + (K_1 + Ka_1 + Ka_2)S^2 + S^3} \quad (23)$$

where E = unmodified enzyme; ES = enzyme with S at the activating site; K_1 = (E)(S)/(ES) = dissociation constant of S from ES; V_1 = maximum initial velocity for the E form; V_2 = maximum initial velocity for the ES form; Ka_1 = Michaelis constant for the E form; Ka_2 = Michaelis constant for the ES form.

Botts and Morales (34) have done a mathematical treatment of this case. Equation 23 is a 3/2 function when written in the reciprocal form.

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